Structural Analyses of Human Basic Fibroblast Growth Factor

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ABSTRACT

The cDNA encoding human basic fibroblast growth factor (bFGF) was expressed in E.coli under the control of a trp promoter. An abundant amount of recombinant human bFGF was highly purified using a heparin affinity HPLC column as described in chapter 4. Upon this chromatography, human bFGF was eluted as four distinct forms which were indistinguishable from each other upon SDS-PAGE under reducing conditions, amino acid composition analysis and partial terminal sequence analysis. The heterogeneity of bFGF upon heparin affinity HPLC was assumed to be derived from the inter- or intra-molecular disulfide linkage of the molecule formed during the purification steps. These molecules showed growth stimulating activity on fibroblast and endothelial cells although their specific activities varied. The angiogenic activity of these molecules was also confirmed.

To study disulfide linkage of the bFGF molecule, each of the four cysteines, amino acid residues 25, 69, 87, and 92, in the mature protein of this factor was individually changed to serine using site-directed mutagenesis as described in chapter 5. The biological activity and heparin binding ability was retained when the serine was substituted for the cysteine residue at either position 69 or 87 of the bFGF protein. These findings indicate that the cysteines at these positions are not essential for the expression of biological activity. Making the substitution at these positions, especially at position 87, reduced the heterogeniety recognized as four peaks of human bFGF eluted from a heparin affinity column, even after oxidation with hydrogen peroxide, suggesting that the cysteines at these positions are exposed to the surface of the molecule and form disulfide bonds

that induce heterologous conformations. In addition, the replacement at position 69 and/or 87 was shown to stabilize bFGF, maintaining its activity.

As described in chapter 6, recombinant human bFGF was used as an antigen to develop, by a somatic cell fusion technique, four monoclonal antibodies (MoAbs) that recognize the complete and amino-terminal truncated form of human bFGF. All these MoAbs bound the complete form of human bFGF produced in E.coli. Competition with synthetic polypeptides, a replicate of amino acids 1-9 and of amino acids 141-146 of human bFGF and truncated forms of human bFGF missing 13 and 40 amino acid residues in the amino-terminal produced in E.coli by recombinant DNA techniques, revealed at least two epitopes recognized by the four IgG type MoAbs. MoAb12 and MoAb78 recognized the epitope located within the first 9 amino acid residues in the amino terminal of complete human bFGF. MoAb52 and MoAb98 recognized the one located between amino acid residue 14 and 40. None of the MoAbs bound bovine aFGF. Using MoAb52 or MoAb98 and MoAb78, a two-site EIA has been developed. This EIA is sensitive enough to detect 0.5 ng/ml of human bFGF. Furthermore, the potential availability of MoAb78 as a ligand for affinity chromatography to purify CS4, which binds weakly to a heparin affinity column, was shown.

The carboxyl-terminal sequence of bFGF is rich in basic amino acid residues, common in the FGF family, and this is considered to greatly contribute to binding to a negatively charged extracellular matrix such as heparin. As shown in chapter 7, to study the relationship between the affinity for heparin and the carboxyl-terminal structure of bFGF, amino- or carboxyl-terminal truncated molecules were produced in E.coli using a recombinant DNA technique. These terminal-truncated bFGFs were applied to a

heparin affinity HPLC column. Truncation of more than 6 amino acid residues from the carboxyl-terminal made the extraction of bFGF from E.coli markedly difficult and weakened the affinity for heparin, though bFGF truncated by up to 46 amino acids showed significant stimulation of DNA synthesis of BALB/c3T3 cells. This stimulation of DNA synthesis was also seen with the bFGF missing 40 amino acids from its amino-terminal, whose affinity for heparin had been shown to be equal to the mature form of bFGF. These results showed the affinity of bFGF for heparin depends significantly on the carboxyl-terminal structure and that the essential part for receptor-binding is present between Asp^{41} and Ser^{100}. Moreover, the important contribution of the sequence Phe^{139}-Leu^{140}-Pro^{141}, which is completely conserved in the FGF family, to the stable structure of the intact molecule is implied.

CONTENTS

LIST OF ABBREVIATIONS

ABTS	2.2'-azino-di[3-ethyl-benzthianozoline sulfonate]
aFGF	acidic fibroblast growth factor
bFGF	basic fibroblast growth factor
bp	base pair(s)
BSA	bovine serum albumin
CAM	chorioallantoic membrane
cDNA	complementary deoxyribonucleic acid
CFA	complete Freund's adjuvant
CL	corpus luteum
CNS	central nervous system
CSF	colony stimulating factor
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EIA	enzyme immunoassay
FBS	fetal bovine serum
FGF	fibroblast growth factor
HRP	horse radish peroxidase
IAA	3-beta-indoleacrylic acid
IFA	incomplete Freund's adjuvant
IL-1	interleukin-1
ip	intraperitoneal(ly)
KGF	keratinocyte growth factor
MMTV	mouse mammary tumor virus
MoAb	monoclonal antibody

mRNA	messenger ribonucleic acid
MRP	mitogen-regulated protein
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	molecular weight
PA	plasminogen activator
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PMSF	phenylmethylsulfonylfluoride
sc	subcutaneous(ly)
SDS	sodium dodecyl sulfate
SIP	superinducible protein
TFA	trifluoroacetic acid
TGF	transforming growth factor
Tris	tris (hydroxymethyl) aminomethane

CHAPTER 1

GENERAL INTRODUCTION

1-1. FINDING OF BASIC FGF

From the late 1960s to the early 1970s several laboratories reported the presence of mitogenic activity in pituitary extracts and in partially purified hormone preparations (Holley and Kiernan, 1968; Clark et al., 1972; Corvol et al., 1972; Armelin, 1973). It was only in 1974, however, that Gospodarowicz elucidated the preliminary characteristics of a fibroblast growth factor (FGF) found in both pituitary and brain (Gospodarowicz, 1974). Within a short period of time, Gospodarowicz (1975) had shown that the molecule present in these acid extracts is a cationic protein (pI>9.6) with a molecular weight (MW) of 14,000-16,000 known as basic FGF (bFGF).

1-2. ACIDIC FGF

In the course of the characterization of bFGF, Gospodarowicz also predicted the presence of distinct activities characterized by neutral or acidic pI, suggesting the existence of structurally distinct classes of mitogens in the brain (Gospodarowicz et al., 1975b). Also, by amino acid analysis Thomas et al. (1980, 1984) characterized aFGF which is biologically related to bFGF and has very similar activity both to the second form of FGF (Gospodarowicz et al., 1975a) and to endothelial cell growth factor (Maciag et al., 1979).

aFGF is 30-100 fold less potent than bFGF (Bohlen et al., 1985) and so far has been found in brain and retina (Thomas et al., 1984; Lobb and Fett, 1984; Bohlen et al., 1985; Baird et

al., 1985a). The mature (140 amino acids) and truncated form (6 amino acid residues removed from the amino terminus) seem to be present in stoichiometric amounts. Since aFGF is less potent than bFGF, it accounts for only 8% and 0.15% of the total mitogenic activity present in crude brain and retinal extracts, respectively; the rest being accounted for by bFGF (Gospodarowicz, 1986; Bohlen et al. 1985).

Comparison of the primary structures has shown that bFGF and aFGF are two very closely related mitogens with a 55% absolute homology(Esch et al., 1985b), suggesting that they are derived from a single ancestral gene (Esch et al., 1985b; Gimenez-Galligo, 1985). As expected from the sequence homology of the basic and acidic forms of FGF and from their ability to support the proliferation of the same spectrum of target cells in vitro, both bFGF and aFGF interact with the same cell-surface receptor (Neufeld and Gospodarowicz, 1986).

The recent cloning of the genes and analysis of cDNA sequences of both bFGF and aFGF suggest that through processes of gene duplication and evolutionary divergence, they have evolved into different gene products (Jaye et al., 1985; Mergia et al., 1986). In humans, the aFGF gene is located on chromosome 5 (Jaye et al., 1985; Mergia et al., 1986), while the bFGF gene is on 4 as mentioned below [1-7].

The nucleotide sequence of cDNA for aFGF appears to code 155 amino acids (Jaye et al., 1985; Abraham et al., 1986a). Proteolytic cleavege of the first 15 residues from the precursor molecule would result in the generation of the mature form (Jaye et al., 1985; Abraham et al., 1986a), but no sufficient hydrophobic core to form a signal peptide is found in the

corresponding region of the aFGF precursor (Jaye et al., 1985; Abraham et al., 1986a).

1-3. FGF RECEPTORS

All cell types that respond to bFGF or aFGF bear specific FGF cell surface receptors. The density of cell surface receptors in normal diploid cells sensitive to FGF is low (10^3-10^4 receptors per cell) (Neufeld and Gospodarowicz, 1985). In the case of established cell lines such as baby hamster kidney (BHK-21) cells (Gospodarowicz et al., 1985b), a higher density of FGF cell surface receptors can readily be detected , and it was with this cell type that FGF receptors were first characterized (Neufeld and Gospodarowicz, 1985, 1986). Binding studies have indicated that the apparent dissociation constant of aFGF binding to BHK-21 cells is $2.5X10^{-10}M$, and approximately $8X10^4$ binding sites are found per cell (Neufeld and Gospodarowicz, 1986). This compares with an apparent dissociation constant for bFGF of $2.7X10^{-10}M$ and $1.2X10^5$ binding sites per cell (Neufeld and Gospodarowicz, 1985). As expected from the high degree of structural homology of bFGF and aFGF, each mitogen can displace the other from its binding sites, suggesting that they bind to the same receptors (Neufeld and Gospodarowicz, 1986). Covalent cross-linking of bFGF or aFGF to their cell surface receptors indicates that both mitogens interact qualitatively with the same two membrane components (MW of 125k and 145k) (Neufeld and Gospodarowicz, 1986). Cell surface bound bFGF or aFGF is, depending on the cell type, slowly internalized (Neufeld and Gospodarowicz, 1985; Olwin and Hauschka, 1986; Moscatelli, 1988).

1-4. FGF FAMILY

The significance of the autocrine growth hypothesis of tumor cells is now further supported by examples of mitogenic autostimulation mediated by some newly discovered members of the FGF family. Three additional members of the family, int-2 (Moore et al., 1986), hst-1 (Yoshida et al., 1987)/KS3 (Delli-Bovi et al., 1987), and FGF-5 (Zhan et al, 1988), have recently been discovered based on the results of various transforming assays designed to identify oncogenes. All five proteins, including bFGF and aFGF, have considerable amino acid sequence homology, ranging from about 35 to 55% identity in commonly aligned regions, and have two similarly positioned introns in their genes. Furthermore very recent work implies the existence of a sixth member of the family, keratinocyte growth factor (KGF) (Rubin et al., 1989).

The first FGF-like oncogene to be identified, int-2, was isolated as one of two transforming genes induced by integration of mouse mammary tumor virus (MMTV) into specific sites in the mouse genome (Smith et al, 1988). This is an example of <u>insertional mutagenesis</u> since the provirus does not code for int-2 but rather integrates adjacent to it so that the expression of this normal mouse gene is amplified by the viral enhancer sequences. Two virtually identical oncogenes, hst-1 (Yoshida et al., 1987) and KS3 (Delli-Bovi et al., 1987), were isolated as transforming genes in an NIH/3T3 transfection assay from a human stomach tumor (hst) and Kaposi's Sarcoma (KS), an AIDS-related tumor usually classified as an angiosarcoma. The hst-1/KS3 gene has subsequently been found in selected hepatomas and a colon tumor. Also, a gene for a protein termed FGF-5 was found in human

bladder carcinoma DNA (Zhan et al., 1988). FGF-5 mRNA has been detected in a second bladder tumor, a hepatoma and an endothelial carcinoma. Finally, KGF was identified in the conditioned medium of a human embryonic lung fibroblast cell line. Purified KGF is a potent mitogen for epithelial cells and capable of stimulating DNA synthesis in quiscent BALB/MK epidermal keratinocytes (>500-fold) with activity detectable at 0.1nM and maximal at 1.0nM. Its lack of mitogenic activity on both fibroblasts and endothelial cells indicated that KGF posseses target cell specificity distinct from that of any previously characterized growth factor. The nucleotide sequence of the cDNA implied that KGF is a distinctive molecule with significant structural homology to the other five members (Rubin et al., 1989).

The correlation between the persistent over-expression by target cells of genes for FGF's with leader sequences and tumor growth is convincing. If these newly discovered FGFs have the wide variety of target cells characteristic of both bFGF and aFGF, then their expression by tumor cells might drive not only tumor growth but also the accompanying hyperplasia of adjacent normal tissue and, perhaps, tumor angiogenesis.

1-5. MOLECULAR STRUCTURE AND CHARACTERISTICS OF BASIC FGF

Complementary DNAs (cDNAs) for bovine and human bFGF have been cloned, and the nucleotide sequence seems to code 155 amino acid residues (Abraham et al., 1986a, b; Kurokawa et al., 1987). On the other hand, structural studies of bFGF suggest that this factor is a single chain polypeptide composed of 146 amino acid residues (Gimenez-Gallego et al., 1986; Klagsbrun et al., 1986). Proteolytic cleavage of the first 9 residues from the precursor

molecule would then generate the mature proteins, and in some cases, a further 15 amino acid residues can then be cleaved (Gospodarowicz et al., 1987). The truncated form of bovine bFGF has the same mitogenic activity as the mature form consisting of 146 amino acid residues. Thus the amino terminal region of bFGF is involved in neither mitogenesis nor binding to cell-surface receptors (Gospodarowicz et al., 1985; Neufeld and Gospodarowicz, 1985, 1986). Because bFGF is thought to function extracellularly, it would be expected to have a signal peptide to direct its secretion. The amino-terminal region of the bFGF precursor (amino acid No.1-13) has the apparent hydrophobic signal peptide core sequence (Esch et al., 1985b); however, there is no evidence from the amino acid sequences of the purified bFGF that signal peptidase cleavage actually occurs at the appropriate position relative to this core (Abraham et al., 1986a), and the hydrophobic core differs from other usual leader sequences of proteins to be secreted by being extremely short (Kurokawa et al., 1987). This is also a feature of interleukin-1 (IL-1) which has no clear signal peptide sequence (Auron et al., 1984). In this respect, bFGF resembles the two forms of IL-1, neither of which have a clear signal peptide sequence (March et al., 1985). After the transfection of COS cells with a human bFGF cDNA clone, growth stimulating activity for BALB/c3T3 cells was detected in the culture medium, suggesting that the nine amino acid region may function as a leader peptide (Kurokawa et al., 1987). However, the mitogenic activity detected in the lysate of DNA-transfected COS cells was ten times higher than that in the culture medium (Kurokawa et al., 1987). So, the nine amino acids may not act sufficiently as a leader peptide, and some regulatory mechanisms may exist for the secretion of bFGF.

Extraordinarily high affinity for heparin, which has been utilized for the isolation and characterization of bFGF from many tissues and tumors, is one of the most prominent characteristics of bFGF. Heparin has been shown to protect bFGF from acid or heat inactivation (Gospodarowicz and Cheng, 1986) and proteolytic degradation (Saksela et al., 1988). The protective effect of heparin on the biological effect of bFGF could have physiological significance. In previous studies, it has been shown that even at 37C and at neutral pH values the in vitro half-life of FGF is only 24h (Westall et al., 1983). Interaction of heparin or hepran sulfate and FGF will likely result in a great extension of FGF's half-life, making low concentrations of bFGF more active.

The presence of FGF receptors on the surface of a wide variety of cells indicates that bFGF must be released from the cells in which it is synthesized. However, since the gene does not appear to code a typical signal peptide sequence (Mergia et al.,1986), it is not known how FGF reaches the cell exterior. This might occur during simple cell lysis (e.g. during tissue injury). Alternatively, it might be achieved by a specialized transport mechanism. Studies have now shown that corneal and vascular endothelial cells actively secreting an extracellular matrix (ECM) which mimics all of the effects of bFGF on cell proliferation and differentiation (Gospodarowicz and Tauber, 1980; Gospodarowicz et al., 1982) contain bFGF (Schweigerer et al., 1987). This could therefore explain the properties of their ECM as well as provide an answer as to how bFGF is released from the cells. Heparin, for which bFGF has high affinity, is closely related to heparan sulfate, which is produced in large quantities by both corneal and vascular endothelial cells, and is

a structural component of the ECM (Gospodarowicz et al., 1986b; Gospodarowicz, 1983). The possibility therefore exists that bFGF could be secreted by the cells in association with the ECM components and become an integral part of the ECM. Indirect evidence for the integration of bFGF into an insoluble substrate such as the ECM can also be derived from the observation that neither media conditioned by capillary nor corneal endothelial cells have a significant impact on cell proliferation (Schweigerer et al., 1987). In contrast, previous studies have shown that seeding those cells on their own denuded ECM will induce them to rapidly proliferate and assume the proper phenotype once confluent (Gospodarowicz et al., 1979, 1982), suggesting that bFGF is not released in a soluble form.

Hydrolysis of the ECM could also result in the liberation of heparan sulfate-FGF complexes which would be biologically active (Gospodarowicz et al, 1986b). In this context, it is interesting to note that during morphogenesis the areas showing the greatest mitotic activity are located where the hydrolysis of the ECM occurs (Bernfield et al., 1984). Similarly, in the case of the embryonic kidney, angiogenesis correlates with the hydrolysis of the kidney mesenchymal stroma (Ekblom et al., 1981). In the adult phase of development, heparan sulfate glycosaminoglycans present in an insoluble form in the ECM can be released from the matrix by heparinase, an inducible enzyme which has been shown to be produced either by platelets when they attach to the subendothelium or by macrophages once they are activated (Matzner et al., 1981; Savion et al., 1985; Yahalom et al., 1984). This could ultimately lead to the solubilization of heparan sulfate-FGF complexes which would be biologically active and could participate in various repair processes, including

wound healing which is described below.

1-6. DISTRIBUTION OF BASIC FGF

A wide distribution of bFGF has been revealed. In fact bFGF is purified from many mesoderm- and neuroectoderm-derived tissues which have been shown to be FGF sensitive either in vitro or in vivo. These include brain, pituitary, kidney, placenta, corpus luteum, adrenal gland, retina, prostate, thymus, bone, the immune system (macrophages-monocytes), and various tumors such as melanoma, chondrosarcoma, and hepatoma(Gospodarowicz et al., 1986b; Baird et al., 1986; Lobb et al., 1986).

Depending on the organ from which it is purified, either the mature (146 amino acids) or truncated (15 amino acid residues cleaved from the amino-terminus) form of bFGF may be present. In the pituitary, brain, and retina, the mature form predominates (Gospodarowicz et al., 1984, 1985b); in the kidney and corpus luteum, only the truncated form can be detected (Gospodarowicz et al., 1985a); while in the adrenal gland and placenta, both forms coexist (Gospodarowicz et al, 1986a). It is not known whether they are artefactually created by specific proteases during bFGF extraction and subsequent isolation.

In addition to these various normal diploid or tumor cells, bFGF has been found in the most unexpected locations. For example, it has recently been reported that the mineralized matrix of osseous tissues harbors abundant mitogenic activity, contributed, at least in part, by bFGF and acidic FGF (See 1-6) (Hauschka et al., 1986). The cellular sources of FGF present in bone matrix have not been defined, but the proliferative effect of bFGF on osteoblasts (Globus et al., 1988) suggests that it

could be locally produced by that cell type and act either as an autocrine or paracrine growth factor. Finally, the localization of bFGF in cell types of unknown function may provide new insights into their physiological role(s). Pituitary glands have long been known to contain a high concentration of bFGF. Recent studies have shown that the main cellular source of bFGF in that organ is the follicular cells which can contain as many as $5X10^5$ bFGF molecules per cell (Ferrara et al., 1987b). This cell type has no endocrine role or well established physiological function, although recent studies have provided evidence for iron transport properties (Ferrara et al., 1987a) and paracrine regulation of hormone secretion (Allaerts and Denef, 1986). Follicular cells are present in large numbers in the pars tuberalis, the most vascularized region of the entire hypophysis. The presence of an angiogenic mitogen such as bFGF in follicular cells might therefore relate to the development and maintenance of the differentiated stage of microvessels. This is consistent with an earlier hypothesis by Harris (1955) who suggested that a major role of the pars tuberalis is providing support for the portal vessels.

The very broad spectrum of activity of FGF suggests that it may serve many unrelated physiological functions. This is in agreement with its wide species distribution ranging from mammalian (Gospodarowicz et al. 1986b; Baird et al., 1986; Kurokawa et al, 1988), avian (Gospodarowicz, 1987), amphibian (Gospodarowicz and Mescher, 1981), and piscean species (Lagente et al., 1986), where bFGF and, to a lesser extent, aFGF seem to be well conserved.

1-7. GENE FOR BASIC FGF

In humans, the bFGF gene is located on chromosome 4 (Jaye et al., 1985; Mergia et al, 1986). The nucleotide sequence of cDNA for bFGF revealed that the primary translation product is composed of 155 amino acids (Jay et al., 1985; Abraham et al, 1986a; Kurokawa et al, 1987). Proteolytic cleavage of the first 9 residues from the precursor molecule would result in the generation of the mature protein.

In various cultured cells and tissues, the bFGF gene encodes two polyadenylated RNAs of approximately 3.7 and 7.0 kilobases (kb) (Abraham et al., 1986b; Schweigerer et al., 1987b, 1987c). The promoters for these two RNAs have not yet been identified. The frequency of bFGF cDNA clones, in various libraries constructed from various tissues known to contain high concentration of bFGF, is extremely low (1 in 5 $X10^5$) and frequently represents unspliced transcripts of the gene. This has led to the proposal that bFGf messenger RNA (mRNA) synthesis *in vivo* takes place at a low level and/or that the fully processed cytoplasmic mRNA is unstable (Abraham et al., 1986b).

The genomic organization of the bFGF gene has been described (Abraham et al., 1986b). Restriction enzyme mapping of the gene indicates that its size is greater than 34 kb. Two introns interrupt the bFGF coding sequence: the first intron splits codon 60 and the second one separates codon 94 and 95.

1-8. EXPRESSION OF BASIC FGF GENE IN MAMMALIAN CELL LINES

Most of the organs from which bFGF has been purified to date have a strong angiogenic potential as well as heavy vascularization in common (Gospodarowicz at al., 1986b). This

suggests that cells of the vascular system might be responsible for bFGF production. Indeed, using the FGF radioreceptor binding assay as well as bFGF-specific cDNA probes or neutralizing antibodies, it was demonstrated that capillary endothelial cells express the bFGF gene and produce the bioactive growth factor (Schweigerer et al., 1987b). In contrast, the cells neither contain nor express aFGF transcripts. This indicates that bFGF could act as an autocrine growth factor for that cell type. The presence of bFGF in capillary endothelial cells also provides an explanation for the rather ubiquitous distribution of bFGF vs aFGF. Recent studies have shown the expression of the bFGF gene and presence of bioactive bFGF in other normal cells, including corneal endothelial cells, lens epithelial cells, adrenal cortex cells, granulosa cells, and osteoblasts (Baird et al., 1986; Schweigerer et al., 1987a, Neufeld et al, 1988a). The same is true for the tumor cells derived from these cells. This has led to the proposal that uncontrolled expression of bFGF may be involved in tumor progression. For example, although the bFGF gene is expressed and bFGF is present in normal adrenocortical cells (Schweigerer et al., 1987a), it must be in a cryptic form, since these cells are nearly completely dependent on exogenous bFGF for proliferation in vitro (Gospodarowicz et al., 1977a). In contrast, the Y-1 adrenocortical tumor cell line, which also expresses and contains bFGF, is not dependent on exogenous bFGF for proliferation in vitro (Gospodarowicz and Handley, 1975). A similar situation exists in the transition from myoblasts to rhabdomyosarcomas (Schweigerer et al., 1987c).

Very recent studies have shown that the expression of the bFGF cDNA in fibroblast cells induces morphological transfomation

and the transformed cells form colonies in soft agar (Sasada et al., 1988; Neufeld et al, 1988b). Moreover, the expression of bFGF fused to some signal peptides in NIH/3T3 cells induces foci of transformed cells at a very high frequency (Blam et al., 1988; Klagsbrun et al., 1988). The transformed cells grow in soft agar and are tumorigenic in nude mice. These results suggest that the cellular transfomation mediated by bFGF is caused by autocrine stimulation with secreted bFGF molecules.

1-9. BIOLOGICAL ACTIVITIES OF BASIC FGF IN VITRO

1-9-1. Effects on cell proliferation

bFGF is a potent mitogen for mesoderm-derived cells (Westermark et al., 1982; Terranova et al., 1985). When tested on vascular endothelial cells derived from either large vessels or capillaries, it triggers cell proliferation at concentrations as low as 2pg/ml, with half-maximal effect occurring at 22.5-39pg/ml (1.5-2.6pM) and saturation at 140-280pg/ml (9.3-18.6pM) (Gospodarowicz et al, 1985b). These concentrations are 20- to 60-fold lower than those required for comparable effects on cell growth or initiation of DNA synthesis with transforming growth factor-beta (TGF-beta), epidermal growth factor (EGF), or platelet derived growth factor (PDGF). bFGF has similar potency in other responsive normal diploid cell types (Gospodarowicz 1985a, 1985b; Gospodarowicz et al, 1985b; Neufeld et al., 1987).

Until now, bFGF has mainly been used in vitro to develop new cell strains (Gospodarowicz, 1979a; Gospodarowicz and Zetter, 1977). bFGF is mitogenic both for cells seeded at clonal density and for low density cultures (Gospodarowicz, 1979a; Gospodarowicz and Lui, 1981). Addition of bFGF to the cultures of most

mesoderm-derived cells results in a greatly reduced average doubling time, which can decrease from 72 to 18 hours. This results primarily from a shortening of the G_1 phase of the cell cycle (Gospodarowicz et al., 1981).

Addition of bFGF to quiescent cultures of Swiss/3T3 cells induced diacylglycerol formation within minutes, protein kinase C activation, and Ca^{2+} mobilization (Tsuda et al., 1985; Kaibuchi et al., 1986). This could correlate with threonine phosphorylation of the FGF receptor, though threonine and serine phosphorylation of the receptor have not yet been examined.

Using cultures of vascular endothelial cells, the early effects of bFGF on membrane motility have been studied (Gospodarowicz et al., 1979b). Within 3 hours both the short- and long-range types of concanavalin A receptor mobility were increased in bovine vascular endothelial cells treated with bFGF. The early effects of bFGF on increased membrane fluidity and increased ruffling activity correlate, as have been shown in the case of EGF (Schlessinger and Geiger, 1981) and PDGF (Westermark et al., 1982), with rapid changes in the dynamic structure of the actin cytoskeleton (Gospodarowicz, 1985b).

The induction of the pleiotypic and mitogenic response by bFGF has been analyzed using confluent cultures of BALB/c3T3 cells. The addition of bFGF to confluent and resting cultures leads to all of the pleiotypic events observed after serum addition (Rudland et al., 1974). Based on data indicating that protein synthesis is required for the rate of DNA synthesis to increase in response to growth stimulation (Rudland et al., 1974), proteins that are specifically synthesized in response to bFGF have been identified. The most dramatic specific increases, after labeling with [^{35}S]-methionine, are in secreted rather than

intracellular proteins. The secreted levels of these proteins begin to rise within 2 hours of the addition of bFGF to growth-arrested cells (Nilsen-Hamilton et al., 1980, 1981; Nilsen-Hamilton and Hamilton, 1982). One of the secreted proteins has been identified as the major excreted protein (Nilsen-Hamilton et al., 1981) and has recently been shown to be a thiol-dependent cathepsin (Denhardt et al., 1986). FGF also stimulates the synthesis and release of a glycoprotein first named mitogen-regulated protein (MRP) (Nilsen-Hamilton et al., 1980). It was later shown that MRP is identical to proliferin (Parfett et al., 1985). It has been proposed that MRP-proliferin could act as an intercellular messenger as either a hormone or paracrine regulator (Nilsen-Hamilton et al., 1980; Nilsen-Hamilton and Hamilton, 1982; Linzer and Nathans, 1984). TGF-beta has been shown to inhibit both the synthesis and release of the major excreted protein and MRP in response to bFGF (Chiang et al., 1986). This correlates with its ability to modulate the biological activity of bFGF. bFGF also increases the secretion levels of a set of five proteins that share the property of being superinduced by cycloheximide. Cycloheximide acts in synergism with bFGF (Hamilton et al, 1985). These cellular proteins have been named superinducible proteins (SIPs). They have MW ranging from 12k (SIP 12) to 62k (SIP 62), and their function is presently unknown (Hamilton et al, 1985).

1-9-2. Effects on cell differentiation

bFGF stabilizes the phenotypic expression of cultured cells. This is a particularly interesting characteristic of bFGF, since it has made possible the long term culturing of cell types that would otherwise lose their normal phenotypes in culture when

passaged repeatedly at a low cell density. This biological effect of bFGF has been best studied using vascular or corneal endothelial cell cultures cloned and maintained in the presence of bFGF and then deprived of it for various time periods (Gospodarowicz et al., 1980; Greenburg et al., 1980; Vlodavsky and Gospodarowicz, 1979; Vlodavsky et al., 1979; Tseng et al., 1982).

Endothelial cells, derived from large vessels or cornea cultured in the presence of bFGF, exhibit at confluence all of the morphological and functional characteristics of the endothelium they are derived from in vivo. When cultured without bFGF, cells at confluence are no longer capable of forming a contact-inhibited cell monolayer with a nonthrombogenic apical cell surface (Greenburg et al., 1980; Vlodavsky and Gospodarowicz, 1979; Vlodavsky et al., 1979; Tseng et al., 1982). However, upon addition of bFGF to the culture medium, the cells reacquire their normal phenotype. This effect of bFGF on cell differentiation correlates with its ability to control processes such as the synthesis and deposition of various ECM components that are known to affect cell surface polarity and gene expression. ECM components whose expression is controlled by bFGF induce synthesis of collagen, fibronectin, and proteoglycans (Gospodarowicz, 1983). Thus, it is possible that one of the ways in which bFGF could promote cell proliferation and stabilization of phenotypic expression is through its effect on ECM production (Gospodarowicz and Tauber, 1980).

bFGF induces capillary endothelial cells to invade the three-dimensional collagen matrix and organize into tubules that resemle blood capillaries. Concomitantly, it stimulates the

endothelial cells to produce a urokinase-type plasminogen activator (PA), a protease that has been implicated in the neovascular response. Thus, bFGF can stimulate processes that are characteristic of angiogenesis in vivo, including endothelial cell migration, invasion, and PA production (Montesano et al., 1986).

When added to chondrocytes (Tseng et al., 1982), bFGF can act as a mitogen as well as a differentiating agent. While costal chondrocytes grown in the absence of bFGF soon assume a fibroblastic appearance and lose their ability to synthesize and release chondroitin sulfate, proteoglycans and collagen type II, cells grown in the presence of bFGF retain these capabilities and at confluence become embedded in a thick ECM which has all of the characteristics of the ECM produced in vivo (Kato and Gospodarowicz, 1985). Interestingly enough, cells will express their correct phenotype only if exposed to bFGF when dividing actively; when added to confluent layers of differentiated and resting chondrocytes, bFGF can no longer reverse their phenotype (Kato and Gospodarowicz, 1985).

It has also been reported that bFGF promotes the differentiation of sheep preadipocyte fibroblasts into adipocytes (Broad and Ham, 1983). A similar observation has been made by Serrero and Khoo (1982) using the 1246 cell line. bFGF also has a pronounced effect on astrocytes, stimulating both their proliferation and the synthesis of glial fibrillary acidic protein (Pettmann et al., 1985; Morrison et al., 1985).

Probably the most spectacular effect of bFGF on cell differentiation is observed in nerve cells. Guroff and co-workers (Togari et al, 1983, 1985) first reported that bFGF acts as a differentiation factor in a rat pheochromocytoma (PC-12) line by

inducing both neurite outgrowth and ornithine decarboxylase activity. Later it was shown that aFGF has similar properties (Neufeld et al., 1987; Wagner and D'Amore, 1986) and that PC-12 cells express specific FGF receptor sites (Neufeld et al., 1987). Similar effects of bFGF on nerve cells have been reported by Walicke et al. (1986) using highly purified populations of fetal rat hippocampal neurons. Under well-defined serum-free cell culture conditions bFGF can increase both neuronal survival and neurite extensions (Walicke et al., 1986). Furthermore, the addition of bFGF to rat cerebral cortical neurons markedly enhances their survival and the elaboration of neurites (Morrison et al., 1986). These results suggest that bFGF may function as a neurotropic agent in the central nervous system.

bFGF regulates expression of cellular oncogenes, in particular that of c-fos and c-myc which have previously been shown to be involved in cell differentiation (Muller and Wagner, 1984) and proliferation (Armelin et al., 1984), respectively. The very rapid appearance of c-fos mRNA and protein after bFGF stimulation strongly suggests that c-fos activation involves a direct effect on gene expression, whereas activation of the c-myc may occur via indirect mechanisms (Muller et al., 1984). The sudden disappearance of c-fos mRNA also contrasts strikingly with the gradual decline in c-myc mRNA (Muller et al., 1984).

Not all of the effects of bFGF on cell differentiation are positive effects. For example, bFGF can delay differentiation and fusion of myoblasts (Gospodarowicz et al. 1976b; Linkhart et al., 1980, 1981). In some established myoblast cell lines , bFGF and aFGF can induce a decrease in creatine phosphokinase expression (Lathrop et al., 1985a, 1985b). These inhibitory FGF effects on

differentiation have been attributed to the ability of FGF to keep myoblast populations in an active proliferative stage, thereby decreasing the percentage of cells in slow growing populations which would enter during their extended G_0, G_1 phase into a stage of terminal differentiation (Lathrop et al., 1985b).

1-9-3. Effects on cell morphology and cell transformation

bFGF has both acute and long term effects on the morphology and growth pattern of responsive cells. Human skin fibroblasts maintained with bFGF in serum-supplemented medium become extremely elongated with long slender projections which are retraction fibrils resulting from an increase in locomotory activity (Gospodarowicz and Moran, 1975). The same is true for vascular endothelial and smooth muscle cells which, in the presence of bFGF, become bipolar (Gospodarowicz, 1985b). Comparison of the locomotor activity of arterial smooth muscle cells and endothelial cells in media supplemented with serum alone or serum and bFGF, respectively, has provided circumstantial evidence that bFGF stimulates random cell migration (Gospodarowicz, 1985b; Terranova et al., 1985). It also has been reported that aFGF could be chemotactic for vascular endothelial cells (Terranova et al., 1985).

Also striking are the effects of bFGF on the morphology of confluent and resting monolayers of BALB/c3T3 cells. Such cells, which show a regular cobblestone pattern in confluent culture exposed to serum-supplemented medium, become spindle shaped and grow in an irregular crisscross pattern when exposed to bFGF for 6-12 hours (Gospodarowicz and Moran, 1974). Large membrane ruffles, often associated with macromonocytotic vesicles, can be seen on most of the cells. Morphologically, the bFGF-treated

cells look transformed since reduced cell-substratum adhesion, growth in crisscross pattern, and increased membrane ruffling are features typical of transformation. The similarity in phenotype of transformed and bFGF-treated cells is consistent with the hypothesis that transformation and bFGF might at least partially share a common metabolic pathway. This effect is reversible since upon removal of bFGF the cells revert to their normal phenotype. bFGF has also been reported to induce the growth in soft agar of nontransformed cells and to potentiate the effect of TGF-beta (Rizzino et al., 1986).

1-9-4. Effects on cell senescence

bFGF significantly delays the ultimate senescence of cultured cells. Addition of bFGF to either clonal or mass cultures of granulosa cells can extend their lifespan from 10 generations to 60 generations (Gospodarowicz and Bialecki, 1978). Adrenal cortex cell lines cloned in the presence of bFGF show a similar dependence on this growth factor during their limited in vitro lifespan (Simonian et al., 1979). Its removal from the culture medium results not only in a greatly extended doubling time but also in rapid cell senescence. In the case of vascular and corneal endothelial cells, bFGF has been shown to extend the lifespan of their cultures (Gospodarowicz et al., 1978a, 1981). Corneal endothelial cells maintained in the absence of bFGF have a lifespan of 20 to 30 generations, whereas in its presence, they can proliferate for 200 generations (Gospodarowicz et al., 1981).

1-10 BIOLOGICAL ACTIVITIES OF BASIC FGF IN VIVO

1-10-1. Mesodermal induction in early embryos

In early embryonic development, the basic body plan arises

because cells in different regions of the egg become programmed to follow different pathways (Thalacker and Nilsen-Hamilton, 1987). During oogenesis, differences arise between the animal and vegetal halves of the eggs. Fertilization results in a subdivision of the vegetal half into a dorsal vegetal and a ventral region of the egg (Slack, 1983; Nieuwkoop, 1969; Smith et al, 1985). This induction is an instructive phenomenon that suppresses epidermal differentiation of cells from the animal pole and directs them instead to differentiate into mesodermal cells. Signal(s) originating from the dorsal vegetal region lead to the formation of the dorsal-type mesoderm, mainly consisting of notochord and somites, while signal(s) originating from the ventrovegetal region lead to the formation of ventral-type mesoderm, consisting primarily of blood cells, mesenchyme, and mesothelium. It has been proposed that this process of regional specification arises from the action of inducing factors, or morphogens. Until recently their chemical nature was unknown, since only minute amounts of the substances that cause mesodermal induction can be isolated from early stage embryo. However, agents that cause induction have been isolated from more abundant sources, such as late stage chick embryo or guinea pig bone marrow and have been characterized as polypeptide (MW 13k) with a basic pI (Tiedemann, 1982; Born et al., 1972).

In recent studies, Slack and his colleagues (Slack et al., 1987) investigated the possibility of bFGF's mimicking the effect of the ventrovegetal signal(s) responsible for the formation of ventral-type mesoderm. When explants of ectoderm cut from the animal pole of stage 8 Xenopus blastulae were exposed to bFGF, the cells, instead of differentiating into epidermis or remaining

undifferentiated, differentiated into mesodermal structures. Between 2 and 30ng bFGF/ml, the induction closely resembled ventral-type mesoderms formed by explants where ventrovegetal regions were combined with animal poles. They consisted of concentric arrangements of loose mesenchyme, mesothelium, and blood cells within an epidermal jacket. At higher bFGF concentrations (30-120ng/ml) most of the explants contained significant amounts of muscle blocks. The inducing effect of bFGF seems to be highly specific, since it could not be mimicked by other growth factors such as TGF-beta or TGF-alpha, tumor necrosis factor, Interferons gamma and alpha, insulin, IL-1 alpha and beta, or colony stimulating factors G-CSF and GM-CSF.

Therefore, in early embryo, bFGF can act as a primordial differentiation factor, inducing the ectoderm to become mesoderm. This is in close agreement with previous in vitro studies which have shown that bFGF has a transforming activity and can act as a morphogen as well as a mitogen on practically all mesoderm-derived cells studied to date. Definite proof that bFGF is the vegetalizing factor and is naturally relevant to mesoderm formation in early embryonic development should be provided by the use of neutralizing bFGF antibodies, which should block induction.

1-10-2. Limb and lens regeneration

Certain amphibian species have a well-balanced capacity to regenerate lost appendages. The dependence of limb regeneration on nerves was reported as early as 1823 by Todd (1823), and studies by Singer (1974) have clarified and extended the concept of neurotrophic control in this process. Denervation of an adult newt limb at the time of amputation or before the formation of

the blastema effectively blocks the regeneration process. If, however, denervation is delayed until after a blastema has formed, differentiation of the blastema cells and morphogenesis of new limb parts occur despite the lack of nerves (Singer, 1974). This finding implies that the neurotrophic effect is needed for the cell proliferation involved in blastema formation but not for the later events of regeneration which include differentiation of blastema cells into muscle, cartilage, and other tissues of the new limb. The molecular basis of the neurotrophic phenomena is poorly understood, but the available evidence suggests that such control is mediated by means of neurosecretory products unrelated to nerve impulses or neurotransmitters (Guttman, 1976).

Blastema cells are thought to originate from either dedifferentiated myoblasts or chondrocytes (Hay, 1974). These are two cell types for which bFGF has been shown to be a potent mitogen (Gospodarowicz et al., 1976b, 1977). Infused bFGF promotes the resumption of mitotic activity in denervated newt limb blastemas in vivo (Gospodarowicz and Mescher, 1981; Gospodarowicz et al., 1978b; Mesher and Gospodarowicz, 1979). When added to cultured blastemas, bFGF at a concentration of 0.1ng/ml is as effective as an optimal dose of brain extract in promoting [^{3}H]-thymidine incorporation (Mescher and Loh, 1980), ^{14}C-labeled amino acid incorporation, and mitotic activity (Carlone and Foret, 1979). Similar concentrations of bFGF have also been shown to maintain the total acetylcholinesterase activity in cultured newt triceps muscle at a level above that in untreated contralateral controls after 1 week (Carlone et al., 1981). In amputated adult frogs (Rana pipiens) the administration of bFGF promotes the formation of heteromorphic regenerating

limbs (Gospodarowicz and Mescher, 1981; Gospodarowicz et al., 1975a) comparable in appearance to those observed by Singer (1974) after surgical augmentation of the nerve supply. These observations lend support to the possibility that bFGF is involved in one of the earlier steps of regeneration, namely recruitment of primitive cells leading to blastema formation.

It has been well-documented for several decades that lens regeneration from the dorsal iris in vivo is dependent upon a stimulus coming from the neural retina (Yamada, 1977; Reyer, 1977). In organ culture, the presence of retina (Yamada, 1974) or pituitary (Connelly et al., 1972; Yamada, 1982) is required for consistent lens regeneration. In both retina (Baird et al., 1985a) and pituitary (Gospodarowicz et al., 1984), the major form of growth factor identified is bFGF, together with smaller quantities of aFGF in the case of retina (Baird et al., 1985a; Courty et al., 1985). bFGF and its agonist, aFGF, could therefore play a role in lens regeneration in vivo. Evidence for this has been presented by Yamada (1982), who reported that crude preparations of retinal derived bFGF could induce lens regeneration in vivo, and by the fact that both retina-derived or pituitary-derived bFGF are potent mitogens for cultured lens epithelial and iris cells (Courty et al., 1985; Gospodarowicz et al., 1977b).

1-10-3. Neurotrophic effects

In addition to being involved in limb regeneration, bFGF could play a role as well in the early development of the nervous system. In vitro bFGF has been shown to be as potent as nerve growth factor in initiating neurite outgrowth from the PC-12 cell line (Neufeld et al., 1987; Togari et al., 1983, 1985;

Wagner and D'Amore, 1986). It is also capable of promoting both the survival and differentiation of nerve cells derived from either the hippocampal region or the cortex (Walicke et al., 1986; Morrison et al., 1986). In addition, nerve cells have been shown to contain bFGF (Pettmann et al.,1986), and preliminary studies have demonstrated that neuronal cell populations are derived from early embryonic brain cholinergic differentiation.

All these effects point toward the possibility of bFGF's playing a role in central nervous system (CNS) development. FGF could also have pronounced effects on CNS astrocytes and oligodendrocytes' proliferation and differentiation (Pettmann et al., 1985; Morrison et al., 1985; Eccleston and Silberberg, 1985) by influencing their properties during normal development or in response to a specific pathogenic event. In addition, bFGF could influence CNS development by way of its angiogenic properties. The early anlagen of the mesencephalon and telencephalon do not contain blood vessels but are surrounded by a primitive perineural vascular plexus (Evans, 1909) from which vascular sprouts radially penetrate the neural tissue at a defined time of development. In the chick, this takes place around day 4 of embryonic development and correlates well with the onset of neuroectodermal proliferation. It has been reported that brain-derived FGF could be responsible for this capillary ingrowth (Connelly et al., 1972). In the embryonic chick brain, FGF-like activity is present in the mesencephalic and telencephalic brain structure from day 3 to day 18 of embryonic development, and the activity increases up to 700-fold in parallel with the increase in neural tissue mass until day 14-16, when the activity reaches a plateau, thereby correlating with the cascade of the ingrowth

of stem vessels into the brain (Risau, 1986).

1-10-4. Wound healing

bFGF, which has been detected in macrophages (Baird et al., 1985b) and after release from damaged cells, could play a crucial role in wound healing processes. In contrast with other growth factors such as PDGF or TGF-beta, bFGF stimulates the proliferation of all the cell types involved in the wound healing process both in vitro and in vivo, including capillary endothelial cells, vascular smooth muscle, fibroblasts, etc., which are involved in the wound healing of specialized tissues. bFGF increases the formation of granulation tissue in vivo (Buntrock et al., 1982a, 1982b, 1984) which is associated with stimulation of the synthetic functions of fibroblasts and myofibroblasts (Buntrock et al., 1984). Similar observations were reported by Davidson et al. (1985) using polyvynyl alchohol sponges implanted sc in rats followed by injection with bFGF. bFGF has also been reported to stimulate the rate of reepithelialization when the epidermis is detached from the dermis and removed after blister induction (Fournier, 1986). In this model, EGF does not increase the rate of wound healing (Fournier, 1986). In specialized tissues such as cartilage, bFGF has been reported to promote chondrossification (Jentzch, 1980), and its presence in bone matrix indicates that it could play an important role in the development and growth of osseous tissue (Globus et al., 1988).

1-10-5. Angiogenic activity

The formation of new blood vessels (angiogenesis) is required for a wide range of developmentally regulated processes occurring in the embryo as well as in the remodelling of tissue occurring

in the neonate or adult. It is also a key feature in pathological situations such as the development of dormant solid tumors, in which capillary invasion is a prerequisite for further growth. A crucial step in the sequential events that lead to the angiogenic response is the invasion of the perivascular ECM by sprouting capillary endothelial cells migration, proliferation, and production of enzymes capable of modifying the ECM. Recently it has become clear that among the growth factors presently described, bFGF and aFGF could be the angiogenic factors controlling capillary proliferation occurring either during organogenesis or during tumor progression (Gospodarowicz et al., 1986).

bFGF has been shown to be a potent angiogenic factor in vivo when tested in assays as different as the rabbit cornea assay, the chick chorioallantoic membrane (CAM) assay, and the hamster cheek pouch assay (Gospodarowicz et al., 1979c,1984, 1985a, 1986). The presence of bFGF as the main mitogen present in corpus luteum (CL) (Gospodarowicz et al., 1985a), adrenal gland (Gospodarowicz et al., 1986), kidney (Baird et al., 1985), and retina (Baird et al., 1985) correlates extremely well with the strong angiogenic properties of these organs.

Angiogenesis plays an impotant role in the development of the CL (Bassett, 1943). This is manifested by the extremely rapid and radical vascular changes that take place in the capillary wreath surrounding the follicle at the time of ovulation. In previous studies (Gospodarowicz and Thakral, 1978) it was demonstrated that rabbit luteinizing granulosa and luteal cells produce a diffusible substance that can trigger the early vascular changes that occur during the development of the CL and

elicits a strong angiogenic response on the part of the host. Similar activities have been described by Jakob et al. (1977), who demonstrated that bovine CL grafted on the CAM, the ventral sc pouch of the mouse, or the hamster cheek pouch elicit a strong angiogenic response. Similarly, Koos and LeMaire (1983) have described gonadotropin-responsive angiogenesis in rat follicles and CL. Frederick et al. (1984), using human follicular fluid , also established the presence of an angiogenic factor that they associated with the perifollicular neovascularization that occurs during folliculogenesis. Taken together, these results suggest that granulosa and luteal cells produce angiogenic factors that trigger capillary invasion into the avascular granulosa cell layer. bFGF, which is present in granulosa cells (Neufeld et al., 1988a), accounts for the full mitogenic potential of CL crude extract (Gospodarowicz et al., 1985a), and is mitogenic for CL-derived capillary endothelial cells (Gospodarowicz et al., 1986), and could , therefore, be responsible for the angiogenic activity observed in the developing CL.

Kiss and co-workers (Kiss and Krompecher, 1962; Kiss and Szabo, 1969) have reported that adrenal extracts are most effective in stimulating vascularization. The isolation of bFGF from adrenal gland extracts, accounting for the full mitogenic potential of the extract, may also account for its angiogenic potential (Baird et al., 1985b). Interestingly, bFGF has also been shown to be mitogenic for adrenocortical cells <u>in vitro</u> (Gospodarowicz and Bialecki, 1978; Simonian et al., 1979). Therefore, the release of bFGF from injured adrenal glands could autostimulate the regeneration of that tissue by triggering proliferation of adrenal cells as well as by triggering capillary invasion, which would bring in nutrients. Also of interest is the

fact that previous reports have described enhanced in vivo chondrossification by crude adrenal gland extract (Kiss and Krompecher, 1962; Kiss and Szabo, 1969). This is in agreement with the fact that bFGF has been shown to stimulate in vitro chondrocyte proliferation (Born et al., 1972; Kato and Gospodarowicz, 1984) and differentiation (Kato and Gospodarowicz, 1985) and to induce in vivo chondrossification (Jentzch et al., 1980).

The retina, where the presence of bFGF and the related agonist aFGF could explain the angiogenic potential of that tissue, as well as a few proliferative retinal disorders, also provides a good example. Over 30 years ago, Michaelson (Michaelson, 1948) proposed the existence of vasculogenic factors produced by the ischemic retina that could induce neovascularization . Although 20 years were required for direct in vitro and in vivo evidence demonstrating angiogenic and endothelial cell-stimulating activity of the retina (D'Amore et al., 1981; Barritault et al., 1981), the original work was pursued by Ashton et al. (1954) and predicted the involvement of the putative vasculogenic factor in the etiology of diabetic retinopathy, retinal fibroplasia, and retinal vein occulusion. With the identification of bFGF as the vasculogenic factor postulated by Michaelson (1948), it is interesting to speculate on the physiological implications of this finding. The effects of FGF on fibroblasts as well as on endothelial cells (Gospodarowicz and Moran, 1975; Gospodarowicz et al., 1976a, 1978a) may explain the association of fibrosis with the neovascularization that is seen in conditions of retrolental fibroplasia and diabetic retinopathy (Gospodarowicz et al., 1978c).

The case of the kidney, where bFGF has been identified as the mitogen which accounts for the full mitogenic as well as angiogenic potential of crude extracts (Baird et al., 1985a), is perhaps one of the most interesting since it would suggest that the appearance of bFGF could be developmentally regulated. Embryonic kidneys strongly stimulate angiogenesis (Michaelson, 1948; D'Amore et al., 1981), and the formation of kidney capillaries is developmentally regulated. Kidney differentiation is driven by interactions between cells of a different developmental history. The epithelial ureter bud induces the differentiation of the mesenchyme and this, in turn, leads to stimulation of blood vessels. The mesenchyme can be stimulated to differentiate in vitro. Previous biological assays suggest that these induced mesenchymes stimulate neovascularization on the CAM (Ekblom et al., 1982; Sariola et al., 1983), and that the blood vessels of the embryonic kidney are of exogenous origin (Sariola et al., 1984). Recently Risau and Ekblom (1986) described the presence of angiogenesis factors in emryonic kidney which are related to bFGF and aFGF (Risau and Ekblom, 1986). Since the invasion of embryonic kidneys by capillaries is both temporally and spatially associated with the formation of pretubular mesenchymal aggregates, it is possible that the expression of FGF within these epithelial structures could be controlled by factors from the epithelial ureter bud which induce differentiation of the mesenchyme (Grobstein, 1956). This, in turn, would lead to stimulation of blood vessel invasion (Sariola et al., 1983; Preminger et al., 1980).

1-10-6. Tumor angiogenesis

Solid tumors are dependent on angiogenesis, since every

increase in tumor cell population must be preceded by an increase in capillary blood supply (Algire et al., 1945; Tannock, 1945). This has lead Folkman and his group (Folkman and Cotran, 1976; Gimbrone et al., 1974) to propose that tumors can release growth factors which induce capillary endothelial cell proliferation and new capillary formation. The isolation of tumor-specific angiogenesis factors from cultured tumor cells or solid tumors has been the subject of intense and unrewarding investigation for the past decade. Since bFGF was isolated in 1974 (Gospodarowicz, 1974), shown to be mitogenic for endothelial cells as early as 1976 (Gospodarowicz et al., 1976, 1978d), and angiogenic in vivo in 1979 (Gospodarowicz, 1979), one must question why bFGF has not been considered a factor in tumor angiogenesis until recently. Three factors probably contributed to the early failure to recognize the potential importance of bFGF. First, it was believed that the angiogenic process was unique to tumors (Ausprunk, 1975), although ample evidence existed that it also occurs in normal tissues (Gospodarowicz and Thakral, 1978). Furthermore, neovascularization of normal tissue was assumed to result from a grafting process of preexisting capillaries within grafted tissues with those of the host (Ausprunk et al., 1975). Second, since bFGF was initially isolated from brain or pituitary, it was thought not to be involved in the tumor angiogenic process. It was also believed that the growth and substrate requirements of endothelial cells derived from large vessels for which bFGF had been shown to be mitogenic were different from those of capillary endothelial cells (Zetter, 1981; Schwartz and Gajdusek, 1982). However, it was later shown that both capillary- and large vessel-derived endothelial cells, irrespective of the organs from which they were derived, have

nearly the same growth and substrate requirements (Gospodarowicz, 1986b). Despite the finding that bFGF was mitogenic for vascular endothelial cells in vitro (Gospodarowicz et al., 1976, 1978d) and angiogenic in vivo (Gospodarowicz, 1979c), it was considered not to stimulate endothelial cell proliferation until as recently as 1984 (Schwartz and Gadjusek, 1982; Maciag, 1984). The realization by Shing et al. (1984) that endothelial cell growth factors have a high affinity for heparin and the use of heparin-Sepharose affinity chromatography for the isolation of these endothelial cell growth factors have led to the purification of the tumor angiogenic factor from solid tumor (chondrosarcoma) and from cultured tumor cells (melanoma, hepatoma) (Lobb et al., 1986; Prats et al., 1989). Once characterized, this factor was found to be similar to bFGF (Klagsbrun et al., 1986). Interestingly enough, the tumor angiogenic factor is an amino-terminal extended form of bFGF that the cloning of bFGF cDNA would have predicted (Abraham et al., 1986b). This extended form has now been shown to be present in normal tissue as well, provided they are extracted in the presence of protease inhibitors (Ueno et al., 1986). The extended form of bFGF has biological properties identical to those of the truncated form. It also seems that in tumors, bFGF is preferentially expressed since numerous neoplastic cell lines have been shown to contain bFGF with apparently little or no aFGF activity present (Gospodarowicz et al., 1986; Moscatelli et al., 1986).

The various loci at which bFGF acts could indicate that it is an important agent in tumor progression. By increasing capillary endothelial cell proliferation, bFGF could be responsible for the incresed vascular supply which delivers O_2 and nutrients and

removes waste products in actively growing tumors. bFGF could also act at the level of the tumor cell itself. By increasing the tumor PA level (Montesano et al., 1986; Mira-Y-Lopez et al., 1986; Moscatelli et al., 1986), as well as by increasing the secreted levels of various proteases (Denhardt et al., 1986) and collagenases (Moscatelli et al., 1986), bFGF would facilitate the metastatic process and tumor invasion. It could also act as a mitogen for tumor cells. In order to proliferate, normal diploid cells are either uniquely dependent on FGF or dependent on various growth factors including FGF, EGF, and PDGF (Gospodarowicz et al., 1978a). In the case of cells depending only on FGF, uncontrolled expression of bFGF in these cells during neoplastic transformation could make them divide in an uncontrolled manner. In the case of cells responding to multiple growth factors, uncontrolled expression of bFGF could make them independent for further growth from an exogenous growth factor supply.

CHAPTER 2

MATERIALS

2-1. COMPLEMENTARY DNA ENCODING HUMAN BASIC FGF

A cDNA clone encoding human bFGF was isolated from a human foreskin fibroblast cDNA library by Kurokawa et al. (1987). The nucleotide sequence of the 4 kb cDNA inserted in the plasmid pTB627 and the deduced amino acid sequence of human bFGF is shown in Fig. 2-1. The amino-terminal amino acid of the mature protein was decided from the published partial amino-terminal amino acid sequence of human bFGF (Gimenez-Gallego et al., 1986). The amino acid sequence of the protein consisting of 146 amino acid residues coincided well with that reported for bovine bFGF. Only two amino acid residues differ: the 112th and 128th amino acid residues of human bFGF are threonine and serine and those of bovine bFGF are serine and proline, respectively. The nucleotide sequence has an open reading frame extending upstrem about 300 bases from the codon for the amino-terminal proline of the mature protein to the 5'-end of the cDNA. But, the extreme GC-rich feature of this upstream region (82 % of the 340 nucleotides are GC) suggests that it may be partly a noncoding sequence. In this region, only one ATG codon is found, and it is in the same translational reading frame as the mature protein, nine amino acids upstream from the amino terminal proline. This ATG is flanked by the sequence (GGACCATGG) that fulfills the Kozak criteria for initiation codons (CCA/GCCATGG) (Kozak, 1984). Therefore, it is suggested that the translation of the precursor molecule may start at this initiator codon ATG as supposed in bovine bFGF cDNA (Abraham et al., 1986a). The 3'-untranslated

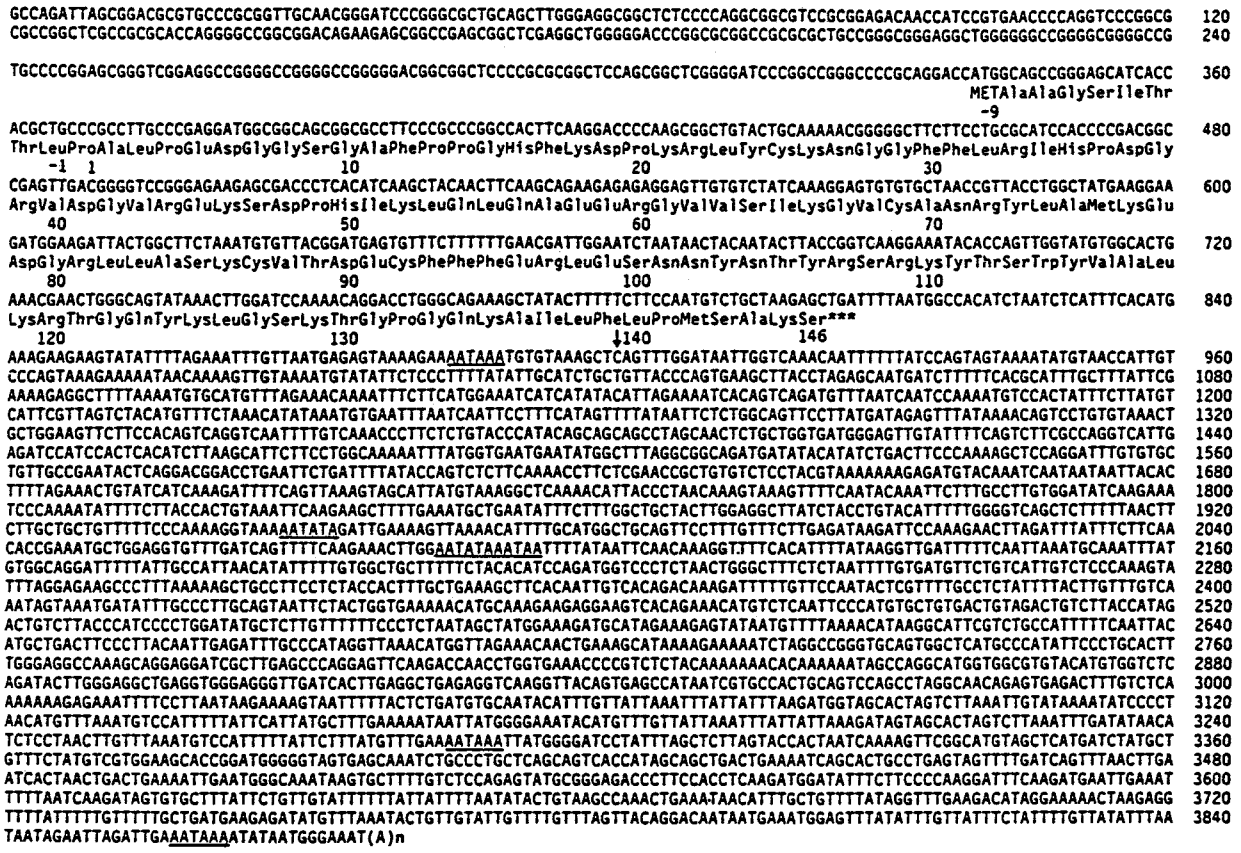

Fig. 2-1. Nucleotide sequence and predicted amino acid sequence of human basic FGF cDNA. The poly(A) additional signal and its analogues are underlined. The position of the start of poly(A) stretch found in a short cDNA (Kurokawa et al., 1986) is indicated by the arrow.

region of the cDNA consists of 3070 nucleotides of an AT-rich sequence. The poly(A) additional signal AATAAA (Proudfoot and Brownlee, 1976) is found 14 nucleotides upstream of the poly(A) homopolymer stretch, while its analogues are present in the 3' non-coding sequence.

2-2. REAGENTS

Restriction enzymes, T4 DNA ligase, E.coli DNA polymerase I, Exonuclease III, Mung-bean nuclease, and bovine pituitary FGF were purchased from Takara Shuzo (Japan). EDTA, 3-beta-indoleacrylic acid (IAA), phenylmethylsulfonylfluoride (PMSF), trifluoroacetic acid (TFA), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dithiothreitol (DTT), egg white lysozyme were from Sigma (USA). Oligonucleotide-directed in vitro mutagenesis system was from Amersham (UK). BALB/c mice for the preparation of ascitic fluid were purchased from Shizuoka-Jitsudoh (Japan). Complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA), and Casamino acid were from Difco (USA). Mineral oil was from Aldrich (USA). Cell culture medium and phosphate-buffered saline were from Flow Labs.(USA). Fetal bovine serum (FBS) was from Micro Biological Associates (USA). GIT serum free medium was from Daigo-Eiyou Kagaku (Japan). Anti-mouse-IgG goat serum conjugated with horse radish peroxidase (HRP) was from Miles Inc.(USA). Bovine aFGF was from R&D systems (USA). Horse radish peroxidase (HRP) was from Boehringer (FGR). The peroxidase substrate kit containing 2.2'-azino-di[3-ethyl-benzthiazoline sulfonate] (ABTS) for the HRP reaction and the Mouse-Typer kit for the mouse Ig isotype identification were from Bio-Rad Labs. (USA). Synthetic polypeptides Pep.1 (a

replicate of the amino-terminal of hbFGF,1-9aa) and Pep.3 (a replicate of the carboxyl-terminal of hbFGF, 141-146aa) were provided by Dr.T.Kikuchi (Tsukuba Research Institute of Takeda Chemical Ind., Japan). Synthetic oligonucleotides were provided by Drs.R.Marumoto and T.Fukuda (Biotechnol. Res. Labs., Takeda Chemical Ind., Japan).

CHAPTER 3

EXPERIMENTAL PROCEDURE

3-1. EXPRESSION OF HUMAN BASIC FGF GENE IN E.coli

3-1-1. Construction of expression plasmid

The cDNA encoding human bFGF was cut out from plasmid pTB627 (Kurokawa et al., 1987) with AvaI and BalI. This DNA fragment was lacking a short 5' portion of the coding region which was filled with a synthetic oligonucleotide having an ATG translational initiation codon. Then the DNA was inserted downstream from the E.coli trp promoter in plasmid ptrp781 (Seno et al., 1986) to construct pTB669 (Fig.3-1).

3-1-2. Preparation of crude extract

E.coli cells were grown at 37C in M9 medium supplemented with 0.4% Casamino acids, 1% glucose and 7ug/ml tetracycline. When the Klett value of the culture medium reached 200, IAA was added to the culture to a final concentration of 30ug/ml, and the culture was continued for a further 4hr (Kurokawa et al., 1983). Bacterial cells were collected by cetrifugation; resuspended in 20mM Tris-HCl (pH 7.6) containing 1mM PMSF and 10% sucrose; placed on ice for 1hr after the subsequent addition of EDTA to a final concentration of 10mM, NaCl to 0.2M and hen egg white lysozyme to 100ug/ml; and treated at 37C for 3 min. The mixture then was sonicated briefly, and the bacterial extract was prepared by centrifugation at 18,000rpm for 40 min (RC-5B, Sorvall, USA).

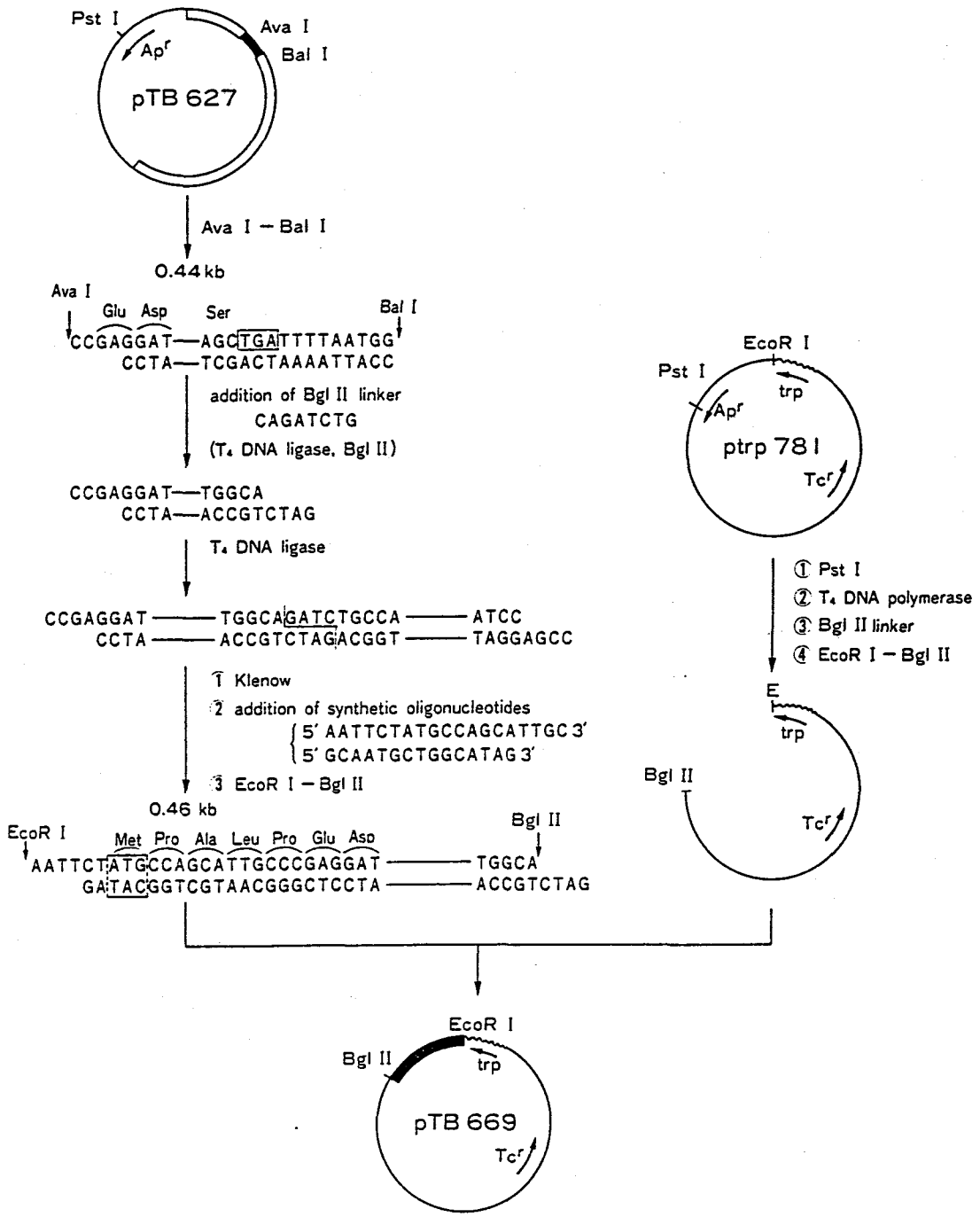

Figure 3-1. Construction of plasmid pTB669 which directs synthesis of human bFGF in E.coli. Plasmid pTB627 contains the human bFGF cDNA inserted into the cloning vector pcDV1 (Okayama and Berg, 1983). The coding region and the 5' and 3' non-translated regions of the cDNA are shown by the solid box and the open box, respectively. The E.coli trp promoter is indicated by the wavy line.

3-2. PURIFICATION OF RECOMBINANT HUMAN BASIC FGF

The extract (25ml prepared from 500ml culture) was applied to a DEAE-cellulose column (DE52, 1X10cm, Whatman, UK) equilibrated with 20mM Tris-HCl (pH 7.6), 1mM EDTA, and 0.2M NaCl. The flow-through fraction (44ml) was collected, and 14ml was applied to a heparin affinity HPLC column (Shodex AF pak HR-894, 0.8X5cm, Showa-denko, Japan). After the column was washed with 20mM Tris-HCl, 1mM EDTA, and 0.5M NaCl, the protein was eluted with a linear concentration gradient of NaCl from 0.5 to 2.0 M in 20mM Tris-HCl (pH 7.4) and 1mM EDTA over 60 min at a flow rate of 1.0ml/min. Reverse phase HPLC column chromatography (Ultron-300 C-4, 0.4X15cm, Chromato Packings Center, Japan) was performed by elution with a linear concentration gradient of acetonitrile from 0 to 90% in 0.1% TFA at a flow rate of 1.0ml/min.

3-3. AMINO ACID COMPOSITION AND TERMINAL SEQUENCE ANALYSES

The amino acid composition of the purified protein was determined on the 24 hr hydrolysate with 5.7N HCl at 110C in the presence of 4% thioglycolic acid. Amino acid analysis was performed on a Hitachi 835 amino acid analyzer (Hitachi, Japan). The amino-terminal amino acid sequence was determined on a gas phase protein sequencer (Model 470A; Applied Biosystems, USA). The carboxyl-terminal amino acid sequence was determined by hydrazinolysis.

3-4. BIOASSAYS

3-4-1. Fibroblast-growth stimulating activity

Growth stimulating activity of bFGF and its derivatives on

mouse BALB/c3T3 cells was assayed using the [^{3}H]-thymidine incorporation method (Maciag et al, 1979). BALB/c3T3 cells were plated at a density of 2x10^3 cells per well on a microtiter plate (96 wells, Nunc, Denmark), and the next day the medium was replaced with 0.2 ml of DME medium supplemented with 0.5% FBS. Twenty hours after the addition of 10ul of sample, [^{3}H]-thymidine (1uCi, 5000Ci/m mol, CEA, France) was added to each well, and after 6 hr, the cells were collected by trypsinization, and the incorporated [^{3}H]-thymidine was counted.

3-4-2. Growth stimulating activity on endothelial cells

Growth stimulating activity on human endothelial cells isolated from umbilical vein (Satoh et al, 1986) was assayed using the modified MTT method described by Tada et al. (1986). Endothelial cells were obtained from human umbilical vein using 0.01% trypsin and 0.02% EDTA in phosphate-buffered saline. The cells were routinely grown in GIT medium containing 2.5% FBS and 2.5ng/ml human bFGF produced in E.coli (medium A). Cells were plated at a density of 2x10^3 cells per well on a microtiter plate (96 wells, Nunc, Denmark), and the next day the medium was replaced with medium A containing the appropriate amount of sample. After 3 days, 100ul of 1mg/ml MTT in medium A was added to each well, and after 4 hr, the produced MTT-formazan was dissolved with 100ul of 10% SDS/ 0.01N HCl, and the absorbance at 590nm was measured to estimate the number of viable cells.

3-4-3. Angiogenesis activity

Angiogenesis activity was monitored on the chick embryo CAM (Vu et al., 1985) and rat cornea (Gimbrone et al., 1974; Langer and Folkman, 1976; Risau, 1986) after grafting polypropylene discs or

Elvax pellets (ethylene-vinyl-acetate polymer, DuPont, USA) containing 6.25 to 200.0ng of bFGF or modified bFGF purified by heparin affinity HPLC.

Note: In the biological assays, FGF purified from bovine pituitary (Takara Shuzo, Japan) was used as the standard.

3-5. PREPARATION OF MONOCLONAL ANTIBODIES

3-5-1. Hybridoma production

Hybridomas to recombinant human bFGF were produced by the procedure of Kohler and Milstein (1975). Six-week-old female BALB/c mice were injected intraperitoneally and in the hind footpads with 10ug of human bFGF in CFA; 10ug booster injections in IFA were given at 3-week intervals. After four injections, 10ug of bFGF in saline was used as a booster. The spleen was removed 5-7 days later, and the splenocytes were fused with P3U1 mouse myeloma cells using 45% PEG 6000. Cells were cultured for 24 hr in 24-well plates ($9X10^5$ cells/well) in IH medium (mixture of Iscove medium and Ham F12 medium in 1:1) containing 20% FBS. The medium was then replaced with IH medium supplemented with 0.1mM hypoxanthine, 0.4uM aminopterin, 16uM thymidine, and 20% FBS (HAT medium). Hybrid cells were screened for using an EIA on day 14. The hybridomas were cloned by limiting dilution.

3-5-2. Antibody production and purification.

Hybridomas which produced monoclonal antibodies were cultured in IH medium with 10% FBS. These cells ($0.5\text{-}1X10^7$ cells) were injected ip into BALB/c female mice that had received 0.5ml of mineral oil ip. After 7-10 days, ascites fluid was collected, and antibodies were purified by ammonium sulfate precipitation and

DEAE cellulose (DE52, Whatman, USA) column chromatography (Staehelin et al., 1981). Further purification was carried out using a hydroxyapatite HPLC column (Mitsui-Toatsu, Japan).

3-5-3. EIA for human bFGF antibodies

Assay plates were coated with 100ul/well of a 5ug/ml solution of human bFGF diluted in PBS by incubating them overnight at 4C. The plates were washed extensively with PBS and incubated with 200ul of 0.1% BSA and 0.01% merthiolate in PBS overnight at 4C. Plates were then washed with PBS, and 100ul aliquots of the hybridoma supernatant were added to duplicate wells. After incubating for 2hr at 25C, the plates were washed with PBS, and 100ul of HRP-labeled goat-antimouse IgG diluted 1 to 10,000 in PBS supplemented with 0.1% BSA was added. After incubating for 2hr at 25C, the plates were extensively washed, and 100ul of peroxidase substrate solution was added to each well. The plates were then incubated for 15 min at 25C. The peroxidase reaction was measured by the increase in absorbance at 415nm.

3-6. TWO-SITE EIA USING MONOCLONAL ANTIBODIES

3-6-1. Preparation of enzyme-labeled antibodies

MoAb 78 (4.2mg), purified using a hydroxyapatite HPLC column, was treated with 0.4mg/ml of S-acetylmercaptosuccinic anhydride for 1hr at 25C to introduce sulfhydryl residues. After Tris-HCl (pH7.0), EDTA, and hydroxylamine, at concentrations of 15mM, 1.5mM, and 150mM, respectively, were added, the reactive MoAb78 was purified by gel filtration using a Sephadex G-25 column (1X80cm) equilibrated with 0.1M Na-phosphate (pH6.0) containing 2.5mM EDTA. HRP was dissolved in 0.1M Na-phosphate (pH6.0) to a concentration of 10mg/1.4ml, and the N-hydroxysuccinimide ester

of N-(4-carboxycyclohexylmethyl) maleimide was added to make 3mg/ml. After incubation at 25C for 1hr, maleimide residues were introduced to HRP, and free reagents were eliminated by gel filtration using a Sephadex G-25 column. These activated MoAb78 and HRP were mixed and coupled by incubation overnight at 4C. MoAb78 conjugated with HRP was purified by an Ultrogel AcA44 column (1X80cm, LKB, Sweden) equilibrated with 0.1M Na-phosphate (pH6.0) containing 2.5mM EDTA at a flow rate of 20ml/hr.

3-6-2. Antibody-sandwich EIA for bFGF and its muteins

Plates were coated with purified MoAb52 or 98 diluted to 20ug/ml with PBS by incubating overnight at 4C. The wells were washed and incubated with 1% BSA in PBS overnight at 4C, and human bFGF diluted with 0.1% BSA in PBS was added. The wells were incubated overnight at 4C and then washed; 100ul of 300-fold diluted MoAb78 HRP conjugates was added. After 4 hr at 25C, the wells were washed extensively, and ABTS was added as a peroxidase-substrate. After incubating for 15 min at 25C, the peroxidase reaction was measured at 415nm.

3-7. PURIFICATION OF DERIVATIVES OF BASIC FGF USING MoAb COLUMN CHROMATOGRAPHY

Purified MoAb78 was coupled to 2ml of Affi-Gel 10 (Bio-Rad Labs., USA) as reported by Staehelin et al. (1981), and a MoAb column equilibrated with 20mM Tris-HCl (pH7.6) containing 1mM EDTA, 0.15M NaCl, and 0.05% NP-40 (buffer A) was prepared. A bacterial extract prepared from 200-ml culture as described in [3-1-2] was diluted 3-fold with buffer A and applied to the MoAb column at 4C at a flow rate of 20ml/hr. The column was washed

successively with 20ml of buffer A and 20ml of buffer A plus 1M NaCl, and then protein was eluted from the column with 20ml of 0.2M Na-acetate (pH4.5) containing 0.2M NaCl and with 20ml of 0.2M acetic acid (pH2.5). The eluate was neutralized quickly with 1M Tris-HCl (pH9.5).

3-8. REPLACEMENT OF AMINO ACID RESIDUES BY SITE-DIRECTED MUTAGENESIS

3-8-1. Site directed mutagenesis of human bFGF cDNA

The EcoRI-BglII portion of plasmid pTB669 [3-1-1], which encodes human bFGF, was inserted into the multicloning sites of bacteriophage M13mp8 (Messing and Vieira, 1982). The single stranded recombinant phage was used as the template in site directed mutagenesis. The procedure of mutagenesis is described elsewhere (Zoller and Smith, 1983; Winter et al., 1982; Taylor et al., 1985). Four oligonucleotide primers were designed to change the cysteine codons at positions 25, 69 ,87 and 92 to serine codons (Table 3-1). Each primer was used independently to induce a single mutation, and each resulting M13 phage was used to substitute more than two cysteine codons with serine codons. At each mutation step, the entire sequence of the gene was verified by dideoxy sequencing (Sanger et al., 1977) to confirm the generated mutations. From each replicative form of the M13 phage having the mutations, the EcoRI-PstI fragment encoding modified bFGF was cut out and integrated downstream from the E.coli trp promoter in ptrp781 (Seno et al., 1986). Finally, plasmids having the genes encoding fifteen types of modified bFGF were prepared and used to transform E.coli MM294 (Backman et al., 1976). Preparation of cell extracts and the procedure for heparin

Table 3-1. SYNTHETIC OLIGONUCLEOTIDE PRIMERS

Four oligonucleotide primers were designed to create serine codons for the codons coding Cys^{25}, Cys^{69}, Cys^{87}, and Cys^{92}. Each mutation is also designed to create a new restriction enzyme site or to eliminate the site for ease in detecting the mutant phage clone. Each mutated nucleotide is indicated by an asterisk (*).

Position of Cys substituted	Sequence of oligonucleotide primer	Modified restriction enzyme site
25	5'CGTTCTTGCTGTAGAGCCGCT3' (* * *)	RsaI
69	5'AACGATTAGCGCTCACTCC3' (* * *)	HaeII
87	5'GTAACAGACTTAGAAGCTAGT3' (* * *)	AluI
92	5'TCGAAGAAAGACTCATCC3' (* * *)	HinfI

affinity HPLC were as described in [3-1-2] and [3-2].

3-8-2. Effect of oxidation reagent

The peak fraction eluted from the heparin HPLC column was dialyzed using distilled water for 3 hr at 4C. The protein was freeze-dried and resolved in 50mM Tris-HCl (pH8.5) and 0.2M NaCl. After BSA to make 1mg/ml and H_2O_2 to make 20mM, used as an oxidation reagent, were added, 200ug of the protein was incubated at 37C for 30 min. After the incubation, the solution was applied to the heparin HPLC column and eluted as described above.

3-8-5. Effect of acidic solvents

bFGF or modified bFGF purified on the heparin HPLC column was diluted to 1ug/ml with 50 mM Na-acetate (pH4.0) and incubated at 37C. At the end of the incubation, the pH was adjusted to 7.0, and the solution was diluted to 100ng/ml of protein with phosphate buffered saline containing 0.5% BSA for the bioassay using mouse BALB/c3T3 cells described in [3-4-1].

3-9. CONSTRUCTION OF TERMINAL TRUNCATED BASIC FGF

3-9-1. Truncation of amino-terminal sequences

Two amino-terminal truncated human bFGFs were produced in E.coli by a recombinant DNA technique (Fig.3-2) and purified by a heparin HPLC procedure as described in [3-2]. Briefly, human bFGF cDNA cloned in phage M13mp8 was modified to trim the amino-terminal 13 amino acid residues of human bFGF by site-directed mutagenesis as described in [3-8-1] using an oligonucleotide primer; this modified cDNA was inserted into ptrp781 (Seno et al., 1986). The plasmid pTB669 DNA was cut with HincII and

ligated with an EcoRI linker including ATG for translational initiation to trim the amino-terminal 40 amino acid residues. E.coli MM294 was transformed with these modified plasmid DNAs, and amino-terminal truncated human bFGFs produced in E.coli were purified using a heparin affinity column.

3-9-2. Truncation of carboxyl-terminal sequence

The EcoRI-BglII portion of plasmid pTB669 [3-1-1], which encodes human bFGF, was inserted into the EcoRI and BamHI sites of plasmid pUC118 (Takara Shuzo, Japan) (Vieira and Messing, 1987), whose HindIII site had been changed to a BglII site using an appropriate synthetic linker, to construct the plasmid pTB1049 (Fig.3-3). This plasmid DNA was digested with XbaI and PstI and was treated with Exonuclease III. The mixture of the resulting single stranded portions of DNA was trimmed with Mung-bean nuclease and then repaired with E.coli DNA polymerase I (Klenow fragment) to make the flush ends complete. By ligating NheI nonsense codon linker (5'-CTAGCTAGCTAG-3') (New England BioLabs., USA) to the flush ends, the mixture of bFGF cDNA having various degrees of deletion at the carboxyl terminus was obtained. From these resulting plasmids, single stranded DNAs were prepared by cotransfecting helper phage KO7 (Vieira and Messing, 1987) and were used as the templates for dideoxy sequencing (Sanger et al., 1977) to determine the truncated region and to verify the entire sequence of the remaining part of the bFGF cDNA. The inserts of these plasmids were transferred to the expression vector pET3c carrying a T7 promoter (Rosenberg et al., 1987).

Each carboxyl-terminal truncated bFGF was produced in E.coli transformed with each plasmid, and the cell extracts were prepared as described in [3-1-2]. The cells lysed with lysozyme

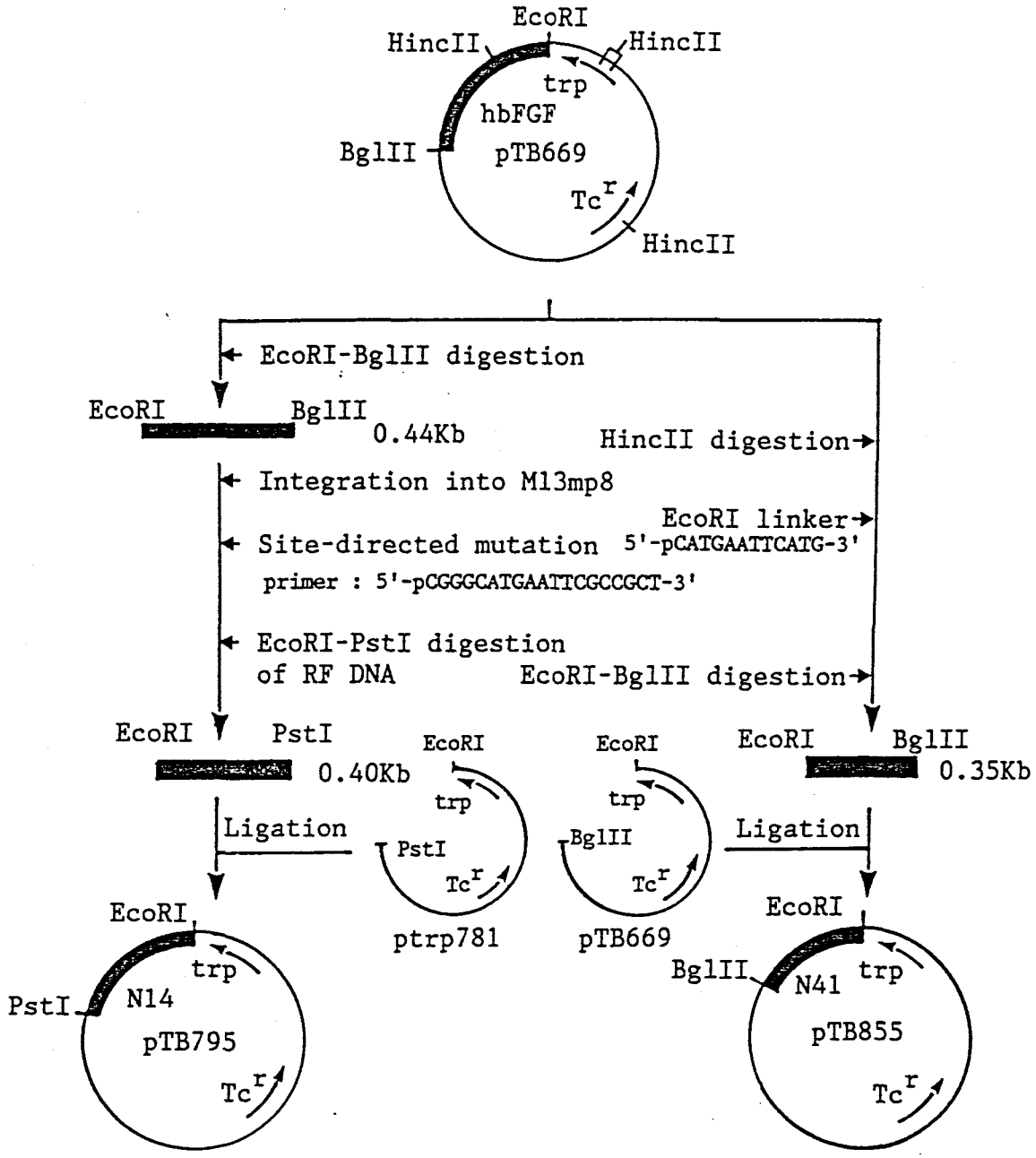

Figure 3-2. Construction of amino-terminal truncated human bFGF. Starting with plasmid pTB669 that directed the synthesis of the complete form of human bFGF under the control of the trp promoter, the plasmid pTB795 was constructed by a site directed mutagenesis procedure with 19-mer of the oligonucleotide primer to produce amino terminal truncated human bFGF, N14, in E.coli. The plasmid pTB855 was constructed from the same original plasmid pTB669 with the insertion of a synthetic EcoRI linker to produce amino-terminal truncated human bFGF, N41.

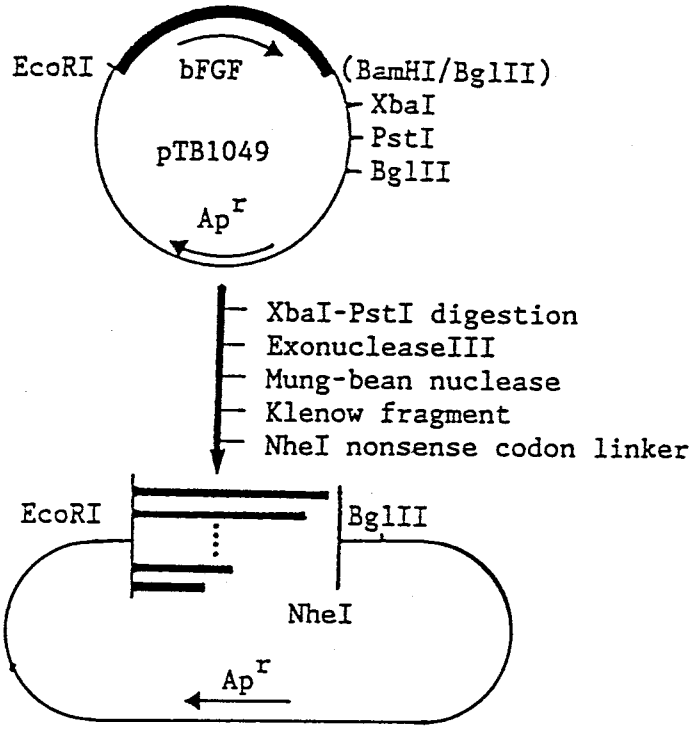

Figure 3-3. Construction of the bFGF cDNA encoding carboxyl-terminal truncated molecules. The EcoRI-BglII DNA fragment encoding mature human bFGF excised from the plasmid pTB669 was inserted into the EcoRI and BamHI sites of plasmid pUC118 (Vieira and Messing, 1987) whose HindIII site had been changed to a BglII site using an appropriate synthetic linker, to construct the plasmid pTB1049. After digestion with XbaI and PstI, this plasmid DNA was treated with Exonuclease III. Exonuclease III is only active on the end created by XbaI digestion under these conditions. The mixture of the resulting single stranded portions of DNA was trimmed with Mung-bean nuclease and then repaired with E.coli DNA polymerase I (Klenow fragment) to make the flush ends complete. By ligating NheI nonsense codon linker (5'-CTAGCTAGCTAG-3') to the flush ends, a mixture of bFGF cDNA having various degrees of deletion at the carboxyl terminus was obtained.

were sonicated briefly in the presence of 5mM DTT, and bacterial cell extract was prepared by centrifugation at 18,000 rpm for 40 min (RC-5B, Sorvall, USA).

The extract from a 100-ml culture of E.coli was applied to a heparin affinity HPLC column (Shodex AF pak HR-894, 0.8X5cm, Showa-denko, Japan), and the protein was eluted with a linear gradient of NaCl as described in [3-2]. Each fraction was assayed using the two-site EIA utilizing monoclonal antibodies against human bFGF [3-6], and the amount of carboxyl-terminal truncated bFGF was estimated.

3-10. SDS PAGE

After boiling intact cells in sample buffer or resolving protein purified by heparin affinity HPLC in the sample buffer, the proteins were analyzed using 17.25% or 20% SDS PAGE (Laemmli, 1970) and Coomassie-Blue or silver staining.

3-11. WESTERN BLOTTING ANALYSIS

Proteins analyzed in acrylamide gels were transferred to nitrocellulose filters in Tris-Glycine buffer for electrophoresis (Laemmli, 1970) containing 20% methanol using a Semi-Dry-electroblotter (Sartorius, FRG). Proteins blotted onto the filter were detected by the monoclonal antibody against human bFGF, MAb78, using the ProtoBlot Western Blot AP starter System (Promega, USA).

3-12. ISOELECTRIC POINT INDEX

The pI value of each amino acid composing the protein

molecules in the FGF family was plotted along with the amino acid sequence position. Each pI value used here is as follows: Asp, 2.77; Asn, 5.41; Thr, 6.16; Ser, 5.68; Glu, 3.22; Gln, 5.65; Pro, 6.30; Gly, 5.97; Ala, 6.00; Cys, 5.07; Val, 5.96; Met, 5.74; Ile, 6.02; Leu, 5.98; Tyr, 5.66; Phe, 5.48; Trp, 5.89; Lys, 9.74; His, 7.59; Arg, 10.76. For the comparison of the members of the FGF family, the position of Cys^{92} in bFGF, which is one of the cysteine residues completely conserved in this family, is set at the same position.

3-13. PREDICTION OF PROTEIN SECONDARY STRUCTURE

The secondary structure of the protein conformation was predicted from the primary amino acid sequence using the procedure described by Garnier (1978).

CHAPTER 4

RECOMBINANT HUMAN BASIC FGF

4-1. INTRODUCTION

Though bFGF is present in a variety of tissues as described in [1-3], it has not been possible to obtain purified protein in abundant amounts even from bovine sources. It has been difficult to obtain a large amount of purified bFGF from human sources. This is the very reason that the studies on the mechanisms of the various functions of bFGF have not been proceeding successfully. Recently, cDNAs for bFGF derived from bovine and human sources were cloned by Abraham et.al. (1986a,b) and Kurokawa et.al. (1987). In this chapter, the construction of the expression plasmid which directs the synthesis of mature type human bFGF in E.coli using the cDNA isolated by Kurokawa et al., the purification of the protein by efficient methods, and the evaluation of the biological activity of the purified protein are described.

4-2. PURIFICATION OF HUMAN BASIC FGF PRODUCED IN E.COLI

4-2-1. Construction of expression plasmid

The cDNA for human bFGF encodes a 155 amino acid precursor molecule. bFGF molecules purified from bovine (Esch et al., 1985a) or human brain (Abraham et al., 1986b) are composed of 146 amino acids, starting at the tenth amino-terminal amino acid of precursor molecule, proline (Giemenez-Gallego et al., 1986). The 9 amino acid residues preceding these 146 amino acids are thought to be processed out after translation (Abraham et al., 1986a). We designed the expression plasmid, pTB669, which directs the

synthesis of the processed mature bFGF molecule in E.coli under the trp promoter. The scheme for constructing pTB669 is shown in Fig.3-1. pTB669 was introduced into E.coli strains, DH-1 (Hanahan, 1983), C600 (Appleyard, 1954) and MM294 (Backman et al., 1976), and the efficiency of the production of human bFGF was examined (Table 4-1). MM294 was found to be the best host cell, producing human bFGF equivalent to 23 mg of bovine FGF per liter of cultue as judged by its mitogenic effect on BALB/c3T3 cells. Therefore, the transformant of MM294 was used to prepare human bFGF.

4-2-2. Purification of recombinant human bFGF

The bacterial extract was subjected to DEAE-cellulose column chromatography. The fraction which passed through the DEAE-cellulose column was applied to a heparin affinity HPLC column, and the proteins were eluted with a linear concentration gradient of NaCl. As shown in Fig.4-1, adsorbed proteins were eluted at four different concentrations of NaCl, namely at 0.95, 1.3, 1.5 and 1.7 M. These were found to be almost homogeneous on SDS-PAGE. They showed a single band at the position of a molecular weight of 17,000 (Fig.4-2). All four fractions showed high mitogenic activity in BALB/c3T3 cells, although the specific activity of each varied (Table 4-2). The p1 fraction, which was eluted first (0.95 M NaCl) and accounted for the major portion of the eluted protein, showed the highest activity. Recovery of the human bFGF activity throughout these purification steps was calculated to be about 37%. The purity of the four human bFGF fractions (p1 to p4) was ascertained by reverse phase HPLC. They each showed a single sharp peak on the chromatograms (Fig.4-3 shows the chromatogram of the p1 fraction), their retention time

Table 4-1. PRODUCTION OF HUMAN BASIC FGF BY SEVERAL E.COLI STRAINS

The mitogenic activity of the extracts from three E.coli strains, DH-1, MM294, and C600, harboring plasmid pTB669 were assayed by measuring the [^{3}H]-thymidine incorporation into BALB/c3T3 cells. The value indicated here is an equivalent value of standard protein on biological activity.

Transformants	Human bFGF (mg/l culture)
E.coli DH-1/pTB669	2.9
E.coli MM294/pTB669	23.2
E.coli C600/pTB669	8.2
E.coli DH-1/ptrp781	<0.0005

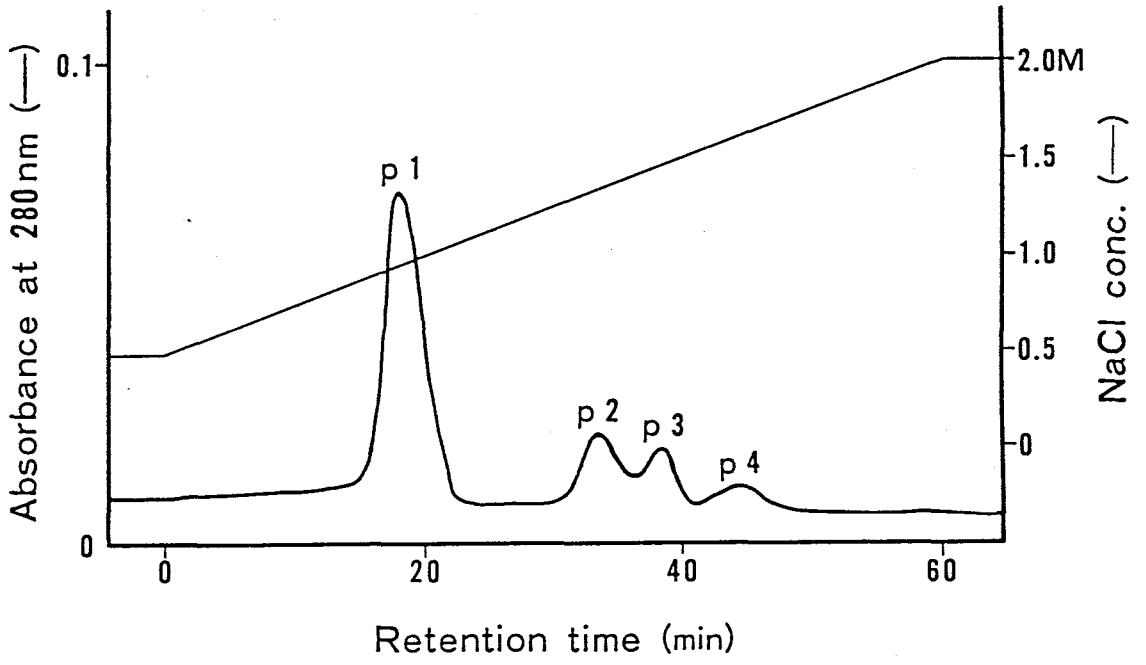

Figure 4-1. Elution profile on heparin affinity HPLC. Thirty milliliters of the flow-through fraction from the DEAE-cellulose column was loaded on the heparin affinity HPLC column (0.8X5cm) and the protein was eluted with a linear concentration gradient of NaCl (0.5-2.0M).

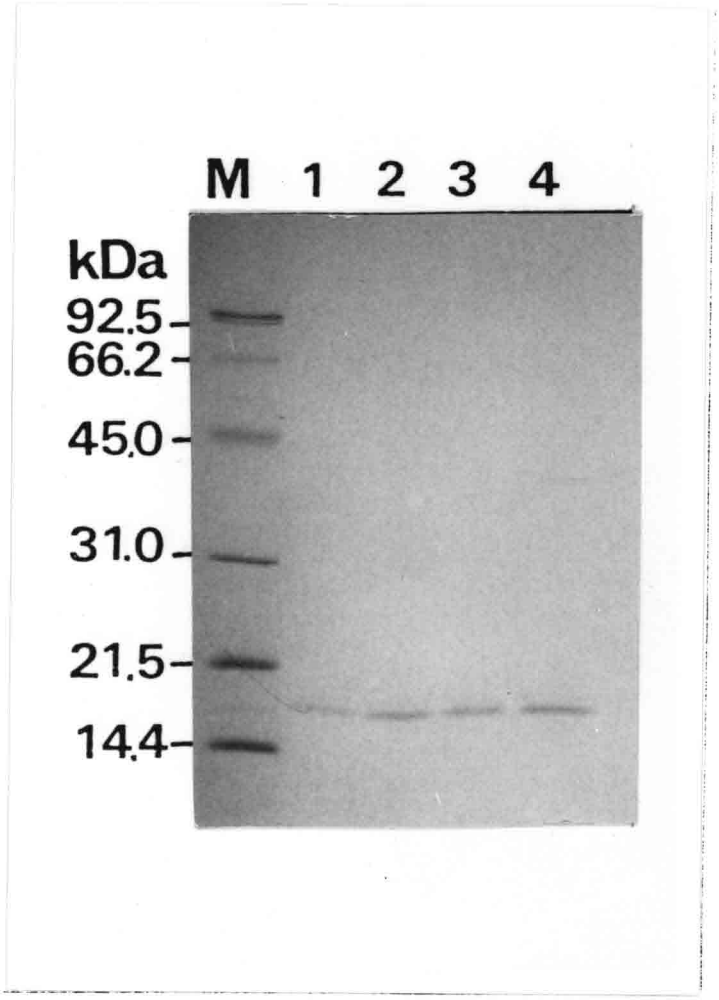

Figure 4-2. SDS-PAGE of heparin affinity HPLC fractions. Electrophoresis was carried out with a 17.25% polyacrylamide gel containing 0.1% SDS under reducing conditions. Two micrograms of protein was loaded on each slot. Lanes M, marker proteins; 1, p1; 2, p2; 3, p3; 4, p4. Proteins were stained with Coomassie-Blue.

Table 4-2. SUMMARY OF PURIFICATION OF HUMAN BASIC FGF

The amount of protein was measured by Bradford's method (1976) with bovine serum albumin as the standard. The specific activity of bovine FGF was given the value of 1.00.

	Total protein (mg)	Specific activity	Recovery (%)
Crude extract	34.2	0.051	100
DEAE-cellulose pass through	29.3	0.032	53
Heparin HPLC			
p1	0.2	2.9	33
p2	0.05	0.66	1.9
p3	0.03	1.03	1.6
p4	0.01	0.34	0.2

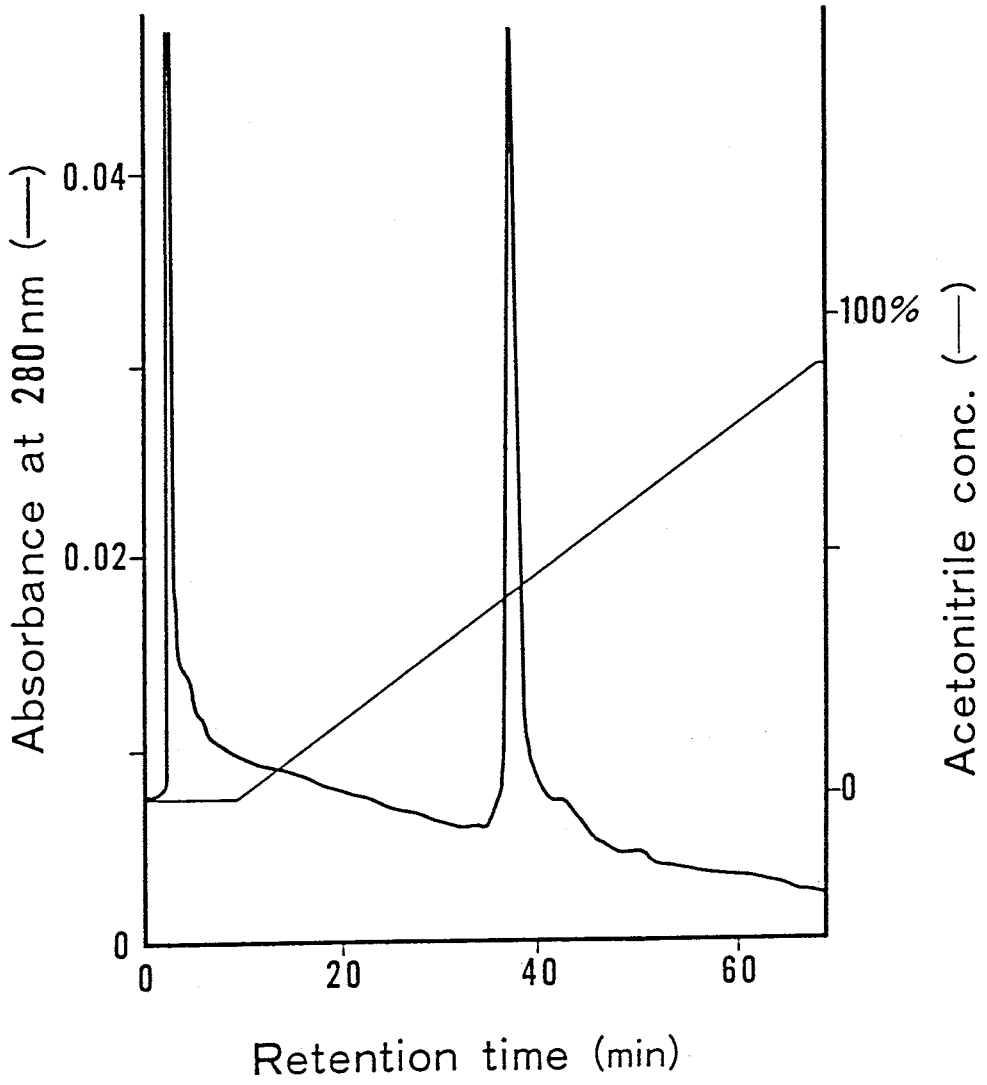

Figure 4-3. Reverse phase HPLC. The p1 fraction from heparin affinity HPLC was applied to a Ultron-300 C-4 column, and the absorbed protein was eluted with a linear concentration gradient of acetonitrile (0-90%) in 0.1% TFA at a flow rate of 1.0 ml/min.

was the same, and they could not be distinguished from each other on the chromatograms. The mitogenic activity of the eluted proteins was completely lost by this chromatographic method because of the effect of the TFA contained in the elution buffer.

4-2-3. Amino acid compositon and partial terminal sequence

The amino acid compositions of the four fractions determined from the analysis of the eluates from the reverse phase HPLC column are shown in Table 4-3. The compositions were the same and agreed well with the values predicted from the cDNA sequence. The sequence of the five amino-terminal amino acids was determined, and the expected sequence (Pro-Ala-Leu-Pro-Glu) was confirmed for the proteins from each fraction. The molecule having methionine at the amino-terminal was detected as less than 15% in each fraction. The carboxyl-terminal amino acid serine was detected by hydrazinolysis of all proteins. Therefore, the four proteins were concluded to be human bFGF molecules composed of the desired 146 amino acids; we were unable to find any differences among them upon the structural analyses performed here.

4-2-4. Preparation under reductive condition

When the bacterial extract was prepared in the presence of the reducing reagent DTT to prevent oxidation of the sample during the preparation steps, proteins were eluted almost exclusively at the p1 position (0.95M NaCl) on heparin affinity HPLC column chromatography (Fig.4-4). Only a very small amount of protein was detected at the p2 position, and no proteins were detected at the p3 or p4 position. The p1 and p2 fractions prepared under these conditions were confirmed to have biological activity.

Table 4-3. AMINO ACID COMPOSITION OF FOUR FRACTIONS OF HUMAN BASIC FGF

Amino acid	p1	p2	p3	p4	Values predicted from cDNA
Asp & Asn	12.0	12.0	12.0	12.0	12
Thr	4.8	4.7	4.7	4.8	5
Ser	8.3	8.6	8.5	8.5	10
Glu & Gln	11.8	12.6	12.5	12.4	12
Pro	8.5	9.3	9.2	9.2	9
Gly	14.7	14.8	14.6	14.7	15
Ala	8.9	9.2	9.1	9.3	9
Cys*	-	-	-	-	4
Val	6.4	6.5	6.7	7.0	7
Met	2.1	2.1	2.1	2.4	2
Ile	3.8	3.9	4.0	4.0	4
Leu	12.6	13.3	13.4	13.4	13
Tyr	6.9	7.1	7.3	7.3	7
Phe	7.8	8.2	8.4	8.5	8
Lys	14.3	13.6	13.8	14.0	14
His	2.9	2.8	3.0	3.2	3
Arg	10.8	11.0	11.0	11.2	11
Trp	1.2	0.9	1.2	1.6	1

* Cysteine was not determined.

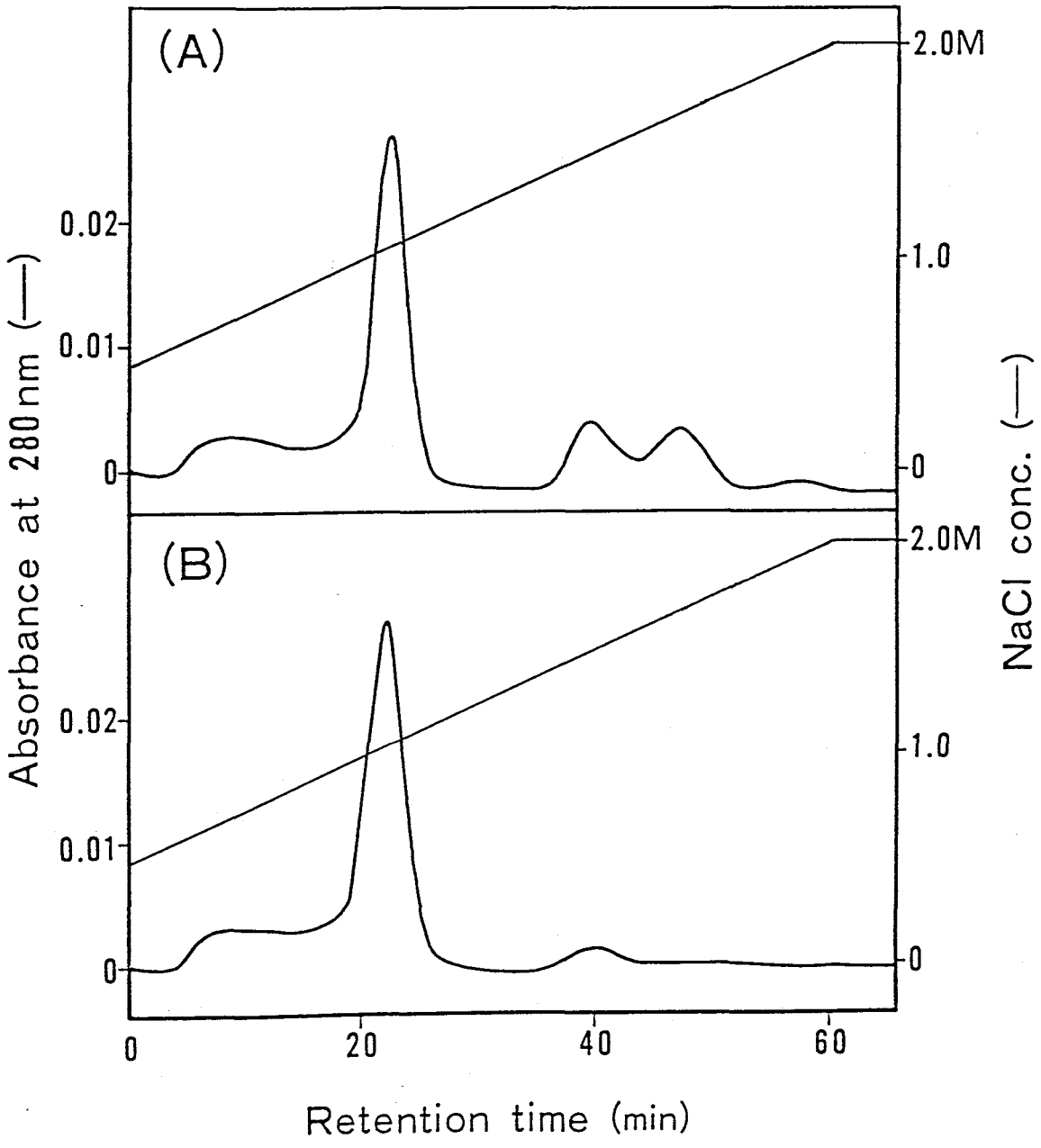

Figure 4-4. Preparation of human bFGF under reducing conditions. Bacterial extract was prepared under non-reducing (A) or reducing (B) conditions. They were applied directly to the heparin affinity HPLC column, and the absorbed proteins were eluted under the same conditions as those described in Fig.4-1.

4-2-5. Biological activity of the purified protein

All of the four fractions, p1 to p4 , were shown to have growth stimulating activity in human endotherial cells and to have angiogenic activity on chick embryo chorioallantoic membrane [Summarized in chapter 5] and rat cornea (Fig.4-5).

4-3. DISCUSSION

Human bFGF synthesized in E.coli could be solubilized and extracted from the cells by a gentle lysis method. For large scale preparation, a French-press was used to disrupt the bacteria instead of the lysozyme-sonication method, because the added lysozyme adsorbed to the heparin affinity column. The crude extract thus prepared was subjected to a CM-cellulose (CM52, Whatman, UK) column chromatography on a column equilibrated with 0.1M phosphate buffer (pH 6.0). After washing the column, the adsorbed protein was eluted with 0.1M phosphate buffer (pH 6.0) and 0.6M NaCl (Bohlen et al., 1984). The eluted protein was applied to a heparin affinity HPLC column (2X25cm). The elution pattern and the purity of the eluate were same as that described in [4-2]. By this method, about 20mg of purified human bFGF could be obtained from 150g of bacterial wet pellet.

The heparin affinity column has been widely used to successfully purify FGF and its family. By this chromatographic method, we were able to elute human bFGF as four peaks. The separated proteins could not be distinguished from each other by the structural analysis described here. The human bFGF molecule has four cysteine residues, which are thought not to participate in sulfhydryl bridging in the native protein (Esch et al., 1985a). We noticed that the elution position of the purified

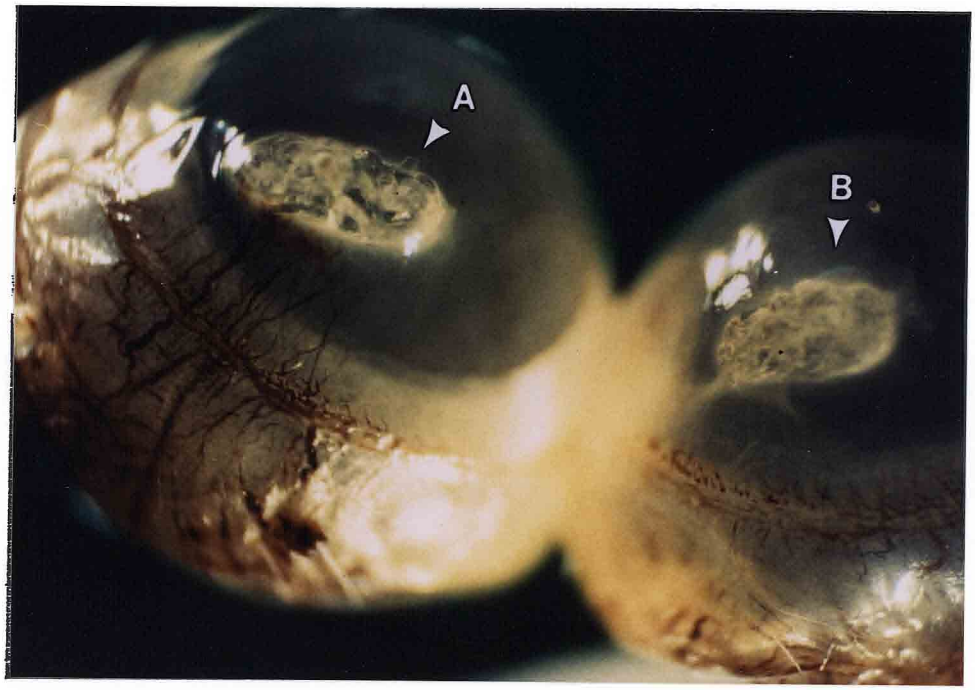

Figure 4-5. Angiogenesis monitored on rat cornea. Vehicle containing 500ng of human bFGF purified from E.coli was implanted into the rat corneal pocket. Photographs were taken under a dissecting microscope on day 14 after surgery. A, recombinant human bFGF (peak 1) 500ng; B, vehicle only as a negative control.

protein from a heparin affinity column shifted to a higher concentration of NaCl after storage of the sample at -20C. When the bacterial extract was prepared under conditions that prevented oxidation of the sample, almost all the human bFGF was recovered at the p1 position. It is interesting that the heparin affinity HPLC column could recognize the minor changes in the conformation of the human bFGF molecule. Studies on the relationship between the heterogeneity and the sulfhydryl bridging in the bFGF molecule are described in chapter 5.

We can now prepare large amounts of human bFGF, and this may be useful for the elucidation of the function of bFGF *in vivo* which is not yet clear.

CHAPTER 5

STUDIES ON THE ROLE OF CYSTEINE RESIDUES

5-1. INTRODUCTION

In chapter 4, the expression of human bFGF cDNA in E.coli and the purification of recombinant human bFGF on a heparin affinity column were described. Through these purification steps, we noticed that bFGF has multiple forms caused by sulfhydryl binding. This heterogeneiety can be reduced by substituting serine for each cysteine through site directed mutagenesis to prevent conformational change, and the stability can be increased without changing the biological activity of bFGF. The biological role of the sulfhydryl bridges in bFGF molcules is not clear yet, but this kind of study using site-directed mutagenesis as described in this chapter will be a good approach to the analysis of the various biological functions of bFGF.

5-2. MOLECULES HAVING CYSTEINE RESIDUES REPLACED WITH SERINE RESIDUES

5-2-1. Replacement of cysteine with serine

Independently utilizing four synthetic oligonucletides as the mutation primers (Table 3-1), each cysteine codon for Cys^{25}, Cys^{69}, Cys^{87}, and Cys^{92} in bFGF cDNA (Fig.2-1) was changed to a serine codon by site-directed mutagenesis (Zoller and Smith, 1983; Winter et al., 1982; Taylor et al., 1985). We also designed primers to introduce or to eliminate some restriction enzyme recognition sites which facilitated the detection of the mutant phage clones (Table 3-1). The mutated DNA coding each type of modified bFGF was inserted into plasmid ptrp781, and fifteen

Table 5-1. MODIFIED BASIC FGFS AND THEIR RELATIVE ACTIVITIES

The plasmids that produce fifteen types of modified bFGFs were prepared, and the modified bFGFs were purified by heparin affinity HPLC. The specific activity of the p1 fraction was determined by the stimulation of DNA synthesis in quiescent mouse BALB/c3T3 cells (Maciag et al., 1979). Specific activity is expressed a value relative to that of bFGF which was assigned a value of 1.0.

Modified bFGF	Plasmid	Substituted position	Specific activity
CS1	pTB739	25	0.4
CS2	pTB742	69	0.9
CS3	pTB743	87	1.0
CS4	pTB744	92	1.0
CS12	pTB776	25,69	0.5
CS13	pTB779	25,87	0.5
CS14	pTB763	25,92	0.3
CS23	pTB762	69,87	1.1
CS24	pTB778	69,92	0.8
CS34	pTB777	87,92	0.5
CS123	pTB764	25,69,87	0.4
CS124	pTB780	25,69,92	0.1
CS134	pTB781	25,87,92	0.5
CS234	pTB782	69,87,92	0.9
CS1234	pTB765	25,69,87,92	0.1
bFGF	pTB669		1.0

types of modified bFGF lacking sulfhydryl radicals originally present in the bFGF molecule were produced in E.coli (Table 5-1).

5-2-2. Heparin affinity HPLC of modified bFGFs

These modified bFGFs were processed by the purification procedure described in [3-2], and the specific elution pattern from the heparin HPLC column was observed for each bFGF derivative (Fig.5-1). With most of the modified bFGFs, the main peak (p1 fraction) eluted from the heparin HPLC column was observed at a retention time of around 20 min. This peak fraction showed a single band on SDS-PAGE. The molecule with the replacement Cys/25/Ser showed a retention time for p1 of a little less than 20 minutes (Fig.5-1). In addition, the molecule with the replacement of either Cys/25/Ser or Cys/92/Ser showed a rather dull and low peak in the elution pattern (Fig.5-1). These observations indicate the loose or weak binding of these types of modified bFGF to heparin. Only three types of modified bFGFs and the original bFGF were efficiently purified using the purification procedure utilizing the heparin column (Fig.5-1).

5-2-3. Growth stimulation of BALB/c3T3 cells by the modified bFGFs

All of the 15 modified bFGFs showed significant growth stimulating activity when assayed using BALB/c3T3 cells (Table 5-1). A decrease in the specific activity of p1 was only recognized in the molecules having Cys/25/Ser replacement (Table 5-1). The three modified bFGFs efficiently purified using the heparin HPLC column showed the same specific activity as the original bFGF when assayed using BALB/c3T3 cells (Table 5-1).

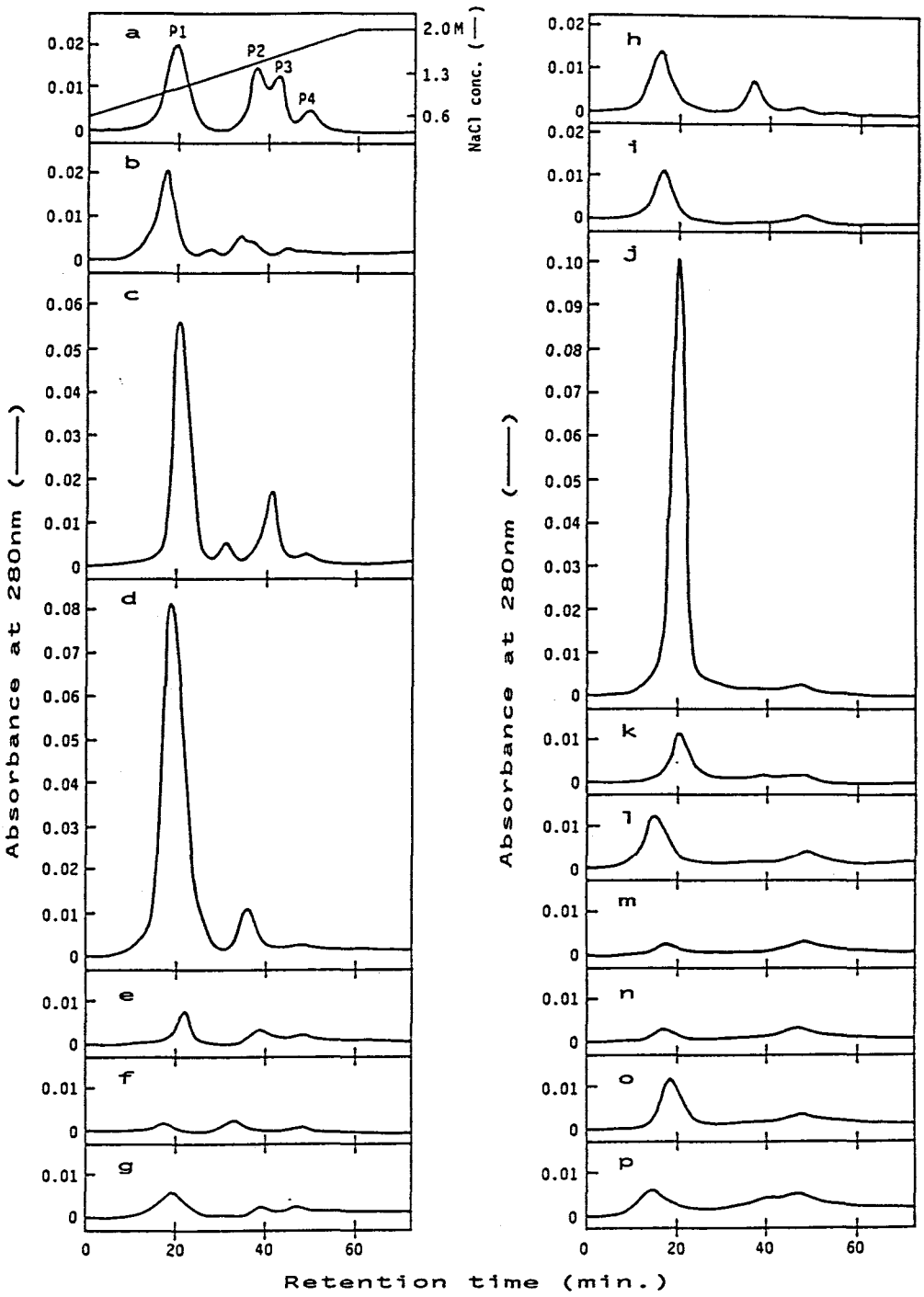

Figure 5-1. Elution profiles of modified bFGFs upon heparin affinity HPLC. The extract prepared from a 500ml culture of each transformant was applied to a heparin affinity HPLC column and the protein was eluted with a linear concentration gradient of NaCl (0.6-2.0M). a; bFGF (control), b; CS1, c; CS2, d; CS3, e; CS4, f;CS14, g; CS24, h; CS12, i; CS13, j; CS23, k; CS34, l; CS123, m; CS124, n; CS134, o; CS234, p; CS1234. The four peaks (p1, p2, p3, p4) recognized in the case of original bFGF are indicated respectively.

5-2-4. Effect of oxidation

CS23 showed a high and sharp peak at a retention time of around 20 minutes on the heparin HPLC column even after being incubated with an oxidizing reagent such as H_2O_2, but with CS2 and CS3, some protein was also detected at a retention time of around 40 minutes (Fig.5-2). These minor peaks are p2 in the case of CS3 and p3 in the case of CS2, both of which are observed in the case of the original bFGF. This fact indicates that the contribution of Cys^{69} and Cys^{87} to the conformation of the original bFGF is reflected in the peaks, p2 and p3, respectively. When a reducing agent, such as DTT, was added after the treatment with H_2O_2, most of the CS2 or CS3 modified bFGF was eluted as a single peak at 20 minutes (data not shown) as was shown with original bFGF [Chapter 4].

5-2-5. Stability under acidic conditions

The stability of the three modified bFGFs (CS2, CS3 and CS23) under acidic conditions was examined. The modified bFGFs were more stable than bFGF in 50mM sodium acetate (pH4.0) at 37C (Fig.5-3). CS23 maintained 50 percent of its activity for at least 20 minutes in this acidic environment, while the original bFGF was inactivated in 10 minutes (Fig.5-3).

5-2-6. Growth stimulating activity in endothelial cells

The efficiently purified bFGFs (CS2, CS3, CS23 and original bFGF) were assayed for their ability to stimulate the growth of endothelial cells isolated from human umbilical vein (Fig.5-4). The modified bFGFs showed almost the same growth stimulating activity in endothelial cells as the original type.

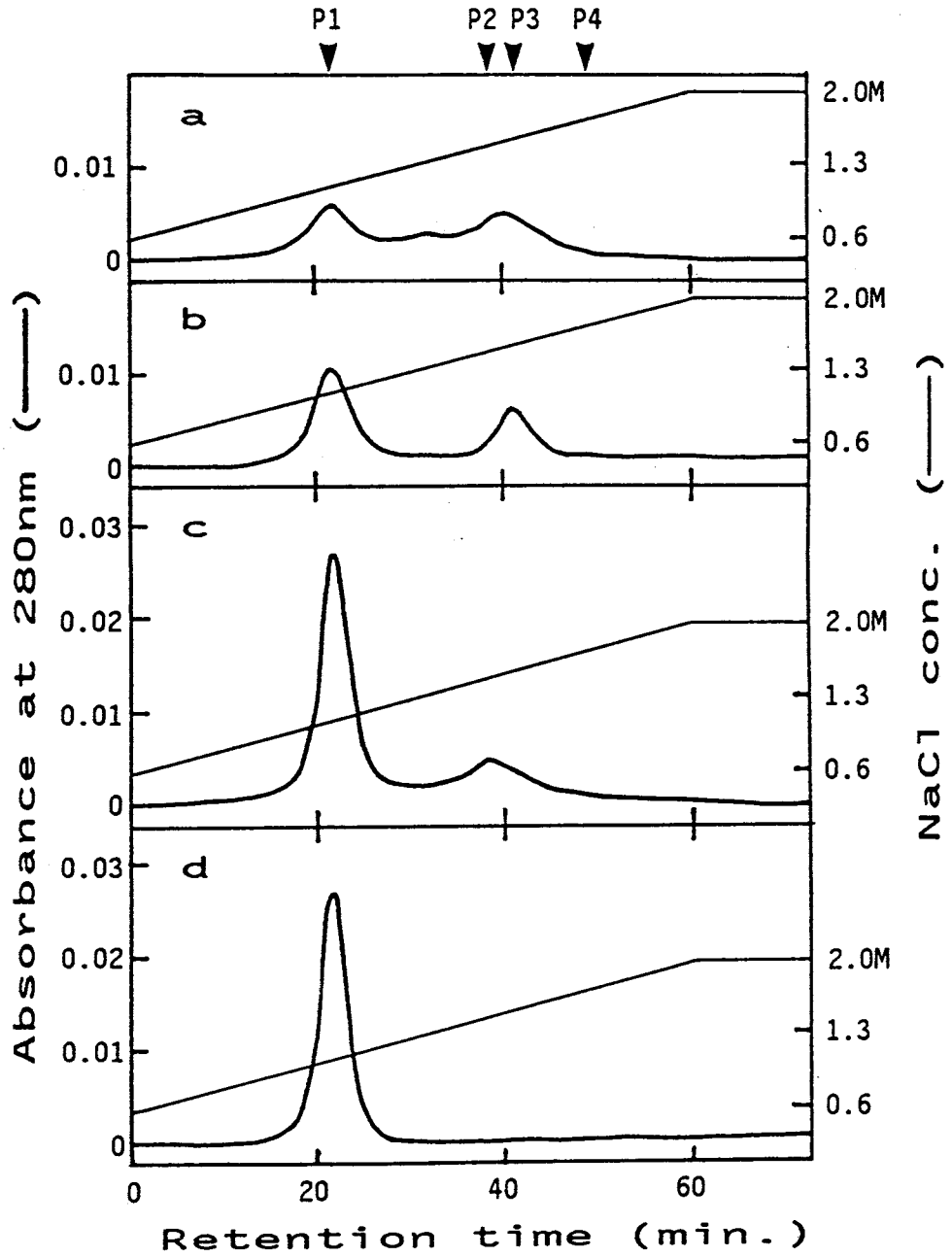

Figure 5-2. Effect of oxidation of modified bFGFs on the elution profiles of heparin affinity HPLC. After appropreate incubation with 20mM H_2O_2, 200ug of the pI fraction of each sample was applied to a heparin affinity HPLC column and eluted as described in Fig.5-1. a; bFGF, b; CS2, c;CS3, d; CS23. The retention time of each peak fraction that appeared in the purification of the original bFGF is indicated by a vertical arrow on the top.

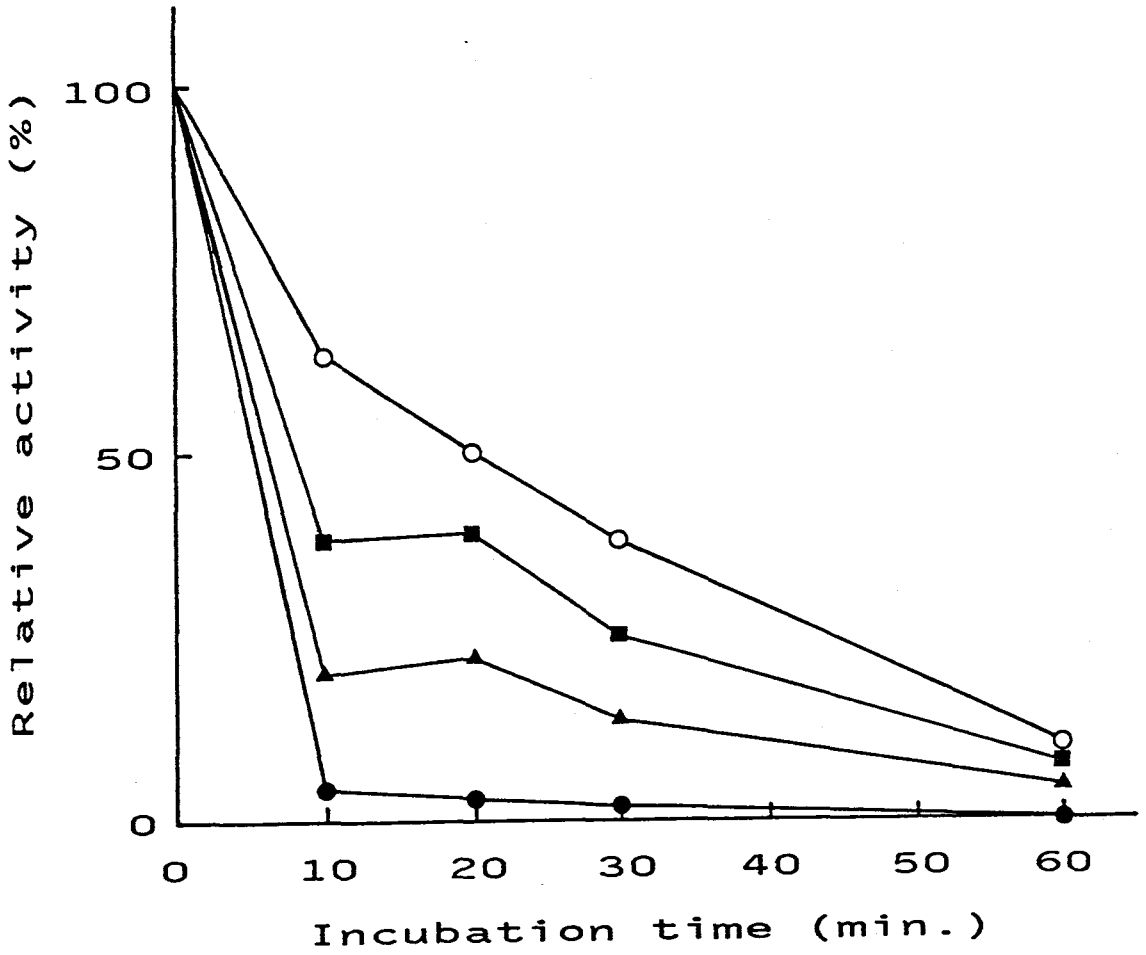

Figure 5-3. Stability of modified bFGFs under acidic conditions. The samples were diluted with 50mM Na-acetate (pH4.0) and incubated at 37C. After appropreate neutralization, they were assayed using mouse BALB/c3T3 cells. The relative activity is calculated with the specific activity at time 0 being 100%. ●; bFGF, ▲; CS2, ■; CS3, ○; CS23. See [3-8-5] in detail for other conditions.

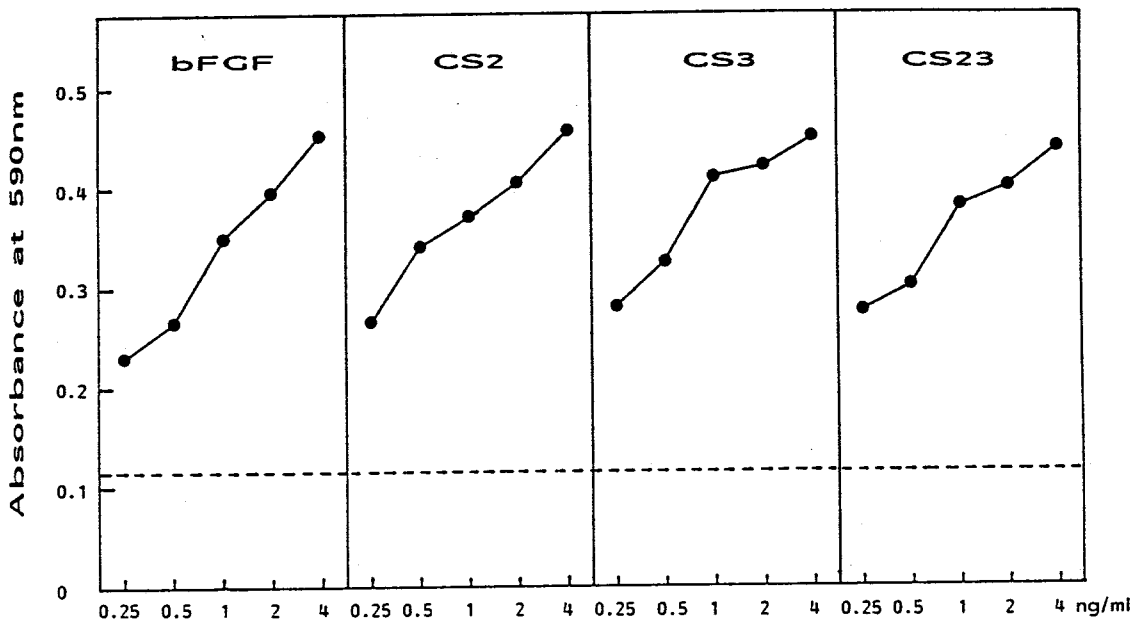

Figure 5-4. Growth stimulating activity in human endothelial cells. Growth stimulating activity of human bFGF and its derivatives was assayed using a modified MTT method (Tada et al., 1986) on human endothelial cells isolated from umbilical vein (Satoh et al, 1986). Samples, bFGF, CS2, CS3, and CS23, were added at concentrations of 0.25 to 4 ng/ml, the MTT-formazan produced was dissolved with 100ul of 10% SDS/ 0.01N HCl, and the absorbance at 590nm was measured to estimate the number of viable cells. The dotted line shows the average level of absorbance measured in the absence of samples. For other conditions in detail, see [3-4-2].

5-2-7. Angiogenic activity of modified bFGFs

The efficiently purified bFGFs (CS2, CS3, CS23 and original bFGF) were also assayed for their angiogenic activity on the chick embryo chorioallantoic membrane (Fig.5-5). The modified bFGFs at 50ng and 12.5ng showed slightly low angiogenic activity compared to that of the original bFGF, but at 25ng the activity was almost same (Fig.5-5A).

5-3. DISCUSSION

Independently utilizing four synthetic oligonucletides as the mutation primers, each cysteine codon in bFGF cDNA was changed to a serine codon by site directed mutagenesis. This type of mutation causes minimal alteration of the structure of the protein because the substitution of an oxygen atom (serine) for a sulfur atom (cysteine) prevents disulfied bond formation at the mutation site. The mutated DNAs coding 15 types of modified bFGF lacking sulfhydryl radicals originally present in the bFGF molecule were expressed in E.coli (Table 5-1).

These modified bFGFs were processed by the purification procedure described for original bFGF [Chapter 4], and the specific elution pattern from the heparin HPLC column was observed for each bFGF (Fig.5-1). With most of the modified bFGFs, the main peak (p1 fraction) eluted from the heparin HPLC column was observed at a retention time of around 20 minutes. The molecule with the replacement Cys/25/Ser showed a retention time for p1 of a little less than 20 minutes (Fig.1). In addition, the molecule with the replacement of either Cys/25/Ser or Cys/92/Ser showed a rather dull and low pattern of elution (Fig.5-1). These observations indicate the loose or weak

A

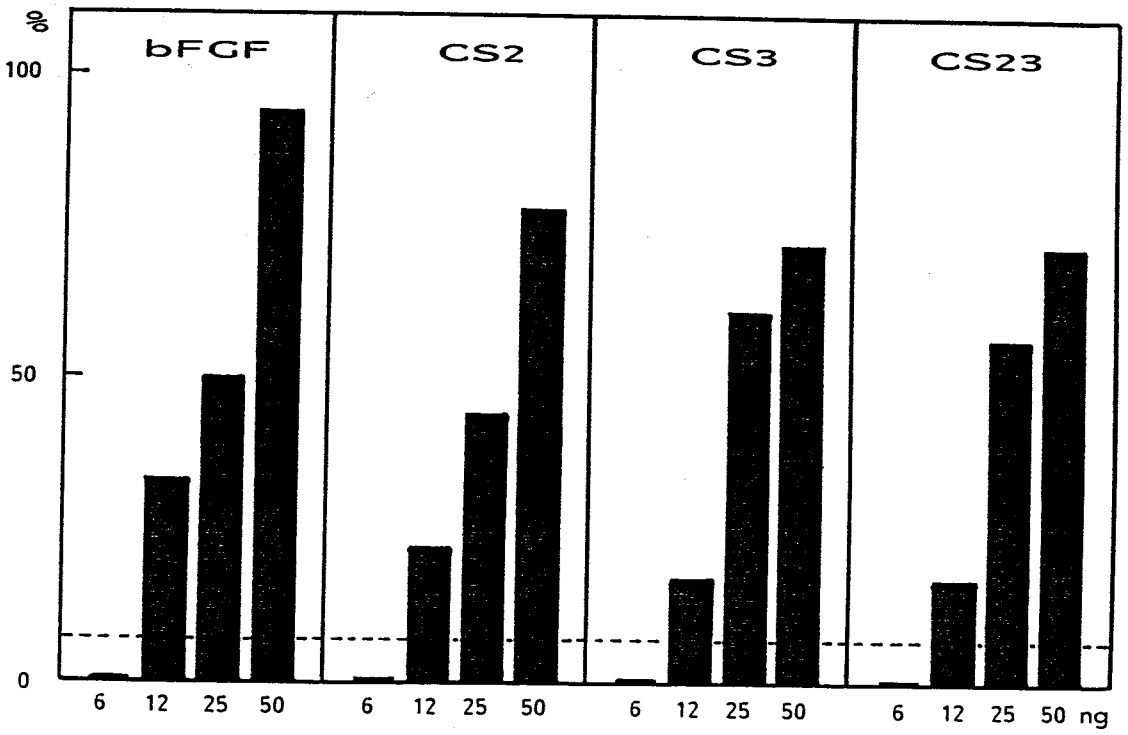

Figure 5-5. Angiogenesis activity monitored on the chick embryo CAM. A, polypropylene discs carrying 6.25 to 50.0 ng of the pl fraction of bFGF, CS2, CS3, and CS23 were grafted onto the membrane. In each case, 18 emblyos were used and the number of embryos that showed angiogenesis toward the disc is presented as a percentage. The dotted line shows the average number of the embryos grafted vehicles only which appeared to show angiogenesis.

B

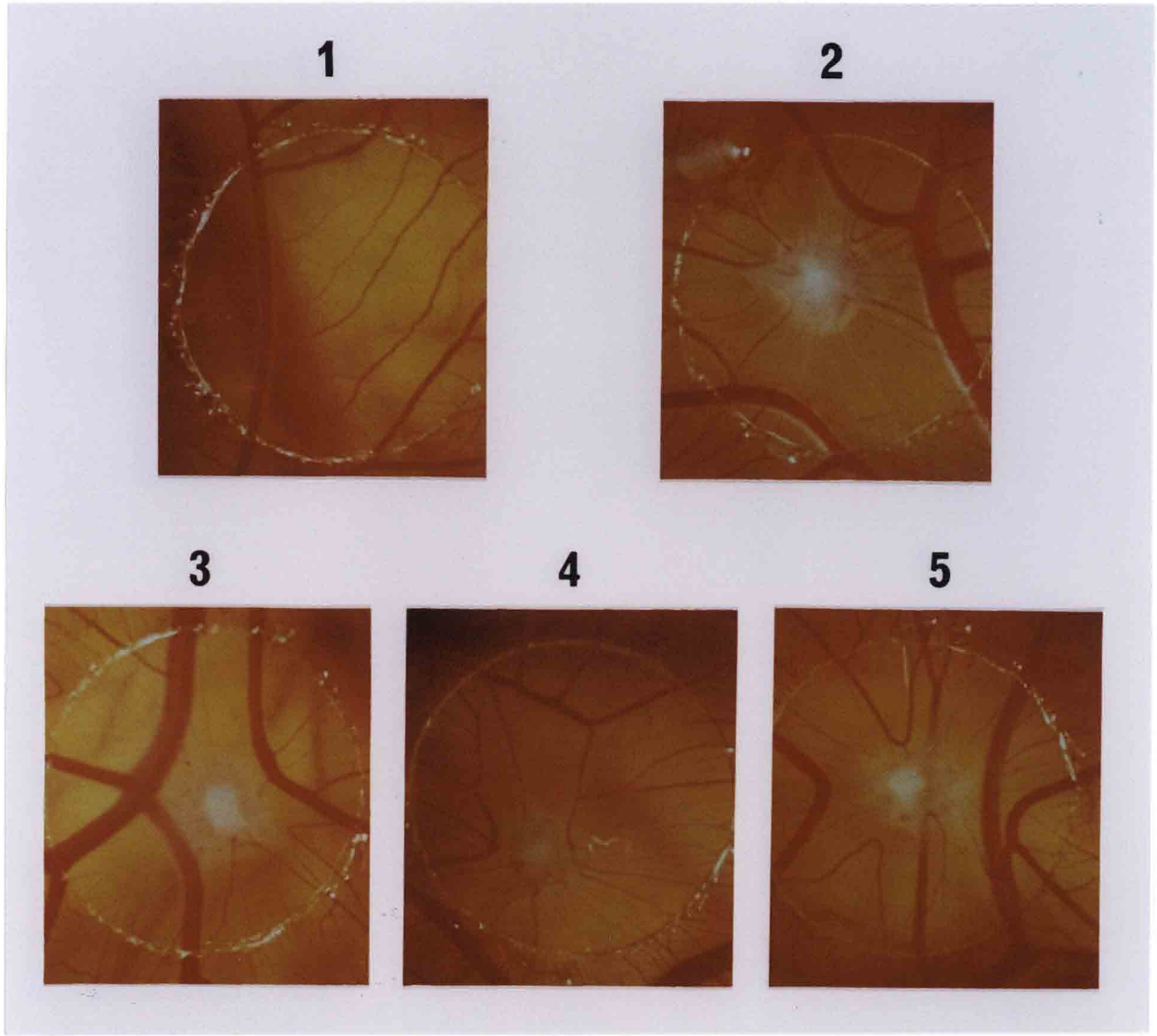

Figure 5-5. Angiogenesis activity monitored on the chick embryo CAM (continued). B, photographs of the chick embryo CAM, on which the discs with 50ng of samples were grafted, taken 14 days after implantation. Plate 1, Vehicle; 2, bFGF; 3, CS2; 4, CS3; 5, CS23.

binding of these types of modified bFGF to heparin. However, a decrease in the specific activity of p1 was only recognized with the replacement Cys/25/Ser (Table 5-1). Therefore, it seems to be difficult to purify the triple or quadruple mutants, which must have a mutation at position 25 or 92, by the method using the heparin affinity column as described for original bFGF [Chapter 4]. The differences among the amounts of modified bFGFs eluted from the heparin column does not seem to be caused by the different affinities of these bFGFs for heparin but seems to be the different solubilities of the molecules from E.coli.

Only three types of modified bFGFs and the original bFGF were efficiently purified using the purification procedure utilizing the heparin column described in [3-2]. These three modified bFGFs showed the same specific activity as the original bFGF (Table 5-1). CS23 showed a high and sharp peak at a retention time of around 20 minutes on the heparin HPLC column even after being incubated with an oxidizing reagent such as H_2O_2, but with CS2 and CS3, some protein was detected at a retention time of around 40 minutes (Fig.5-2). These minor peaks are p2 in the case of CS3 and p3 in the case of CS2, both of which are observed in the case of the original bFGF. This fact indicates that the contribution of Cys^{69} and Cys^{87} to the conformation of the original bFGF is reflected in the peaks, p2 and p3, respectively. When a reducing agent, such as DTT, was added after the treatment with H_2O_2, most of the modified bFGF CS2 or CS3 was eluted as a single peak at 20 minutes (data not shown) as was shown in the case of original bFGF [Chapter 4]. These results indicate that the cysteine residues at 69 and 87 are not responsible for the affinity to heparin and that they are exposed to the surface of the bFGF molecule to form the intra- or

intermolecular sulfhydryl bridging, which results in several heterologous peaks on heparin affinity chromatography. The absence of cysteine residues at both 69 and 87 protects the bFGF molecule from the oxidization of the sulfhydryl residues.

Westall et al. reported that bFGF from bovine brain is inactivated by incubation in an acidic solvent (Westall et al., 1983). We have tested the stability of modified bFGFs under acidic conditions. The modified bFGFs (CS2, CS3 and CS23) were more stable than bFGF in 50mM sodium acetate (pH4.0) at 37C. CS23 maintained 50 percent of its activity for at least 20 minutes in this acidic environment, while the original bFGF was inactivated in 10 minutes (Fig.5-3).

We then examined these modified bFGFs to see whether or not they carry the same biological activity as the original bFGF; the efficiently purified bFGFs (CS2, CS3, CS23 and original bFGF) were assayed for their ability to stimulate the growth of endothelial cells (Fig.5-4) and for their angiogenic activity on the chick embryo chorioallantoic membrane (Fig.5-5). The modified bFGFs showed growth stimulating activity on endothelial cells almost equal to that of the original type (Fig.5-4). The modified bFGFs at 50ng and 12.5ng showed slightly low angiogenic activity compared to that of the original bFGF, but at 25ng the activity was almost same (Fig.5-5). It can be concluded that the activity on the growth of endothelial cells and on angiogenesis is conserved in the modified bFGFs at almost the same level as the activity of the original bFGF.

We have described here the modification of human bFGF by the substitution of cysteine residues with serine residues. We have also described the elution profiles from a heparin affinity

column, stability under acidic conditions and the biological activity of three highly purified modified bFGFs (CS2, CS3 and CS23). These analyses indicate that Cys^{69} and Cys^{87} of bFGF are not essential for its biological activity; these residues mainly participate in sulfhydryl bridging which induces heterogeneiety in the elution profiles upon heparin affinity column chromatography. When the amino acid sequences of basic and acidic FGF (Gimenez-Gallego et al., 1985; Esch et al., 1985b; Jaye et al., 1986) are compared, only Cys^{25} and Cys^{92} are conserved; therefore, the heterogeneiety in conformation is considered to be a specific character of bFGF, and the reason for the difference in biological activity of these two proteins may lie in this point. Furthermore, these two cysteine residues are well conserved even in oncogenes, int-2, hst-1, KS3, and FGF-5, whose amino acid homologies to FGF have been reported recently (Moore et al., 1986; Dickson and Peters, 1987; Yoshida et al., 1987; Delli-Bovi et al., 1987; Zhan et al., 1988) implying the angiogenic function of these oncogene products. These facts imply that these two cysteine residues are essential for the basic functions of the proteins in the FGF family: affinity for heparin and growth stimulating activity in fibroblasts.

As a result of the modifications described here, we have succeeded in effectively engineering bFGF. This success demonstrates bFGF's resistance to antecedent oxidation and the stability of its biological activity under acidic conditions. These modified bFGFs are expected to be developed for clinical application as accelerated remedies for injuries, burns and thrombi.

CHAPTER 6

MONOCLONAL ANTIBODIES AGAINST HUMAN BASIC FGF

6-1. INTRODUCTION

Depending on the organ from which bFGF is purified, either the mature or truncated form of bFGF can be detected. In the pituitary and brain, the mature form predominates (Gospodarowicz et al., 1984, 1985b); in the kidney and corpus luteum, only the truncated form can be detected (Gospodarowicz et al., 1985a); while, in the adrenal gland, both forms coexist (Gospodarowicz et al., 1986a).

cDNA for human bFGF has been cloned, and the nucleotide sequence seems to code 155 amino acid residues (Abraham et al., 1986b; Kurokawa et al., 1987). On the other hand, partial structural studies of the amino acid sequence suggest that human bFGF is a single chain polypeptide composed of 146 amino acid residues (Gimenez-Gallego et al., 1986, Klagsbrun et al., 1986). Proteolytic cleavage of the first 9 residues from the precursor molecule would generate the mature protein. A further 15 amino acid residues can then be cleaved in some tissues (Gospodarowicz et al., 1987). The truncated form of bovine bFGF is as mitogenic as the mature form consisting of 146 amino acids. Thus the amino terminal region of bFGF is involved in neither mitogenesis nor binding to cell-surface receptors (Gospodarowicz et al., 1985a; Neufeld and Gospodarowicz, 1985, 1986).

Comparison of the primary structures have shown that bFGF and aFGF, which is 30-100 fold less potent than bFGF (Bohlen et al., 1985), are two very closely related mitogens with 55% absolute amino acid homology (Esch et al., 1985b), suggesting that they are derived from a single ancestral gene (Esch et al., 1985b;

Gimenez-Gallego et al., 1985). As expected from the sequence homology of the basic and acidic forms of FGF and from their ability to support the proliferation in vitro of the same spectrum of target cell, both bFGF and aFGF interact with the same cell-surface receptor (Neufeld and Gospodarowicz, 1986).

Very recently the oncogenic character of FGF has been pointed out by the highly conserved homology in the FGF family. In mammalian cells, the expression of bFGF cDNA results in the transformation of the host cells (Rogelj et al., 1988; Sasada et al., 1988). These findings may imply that FGF has the characteristics of an active oncogene product including angiogenic activity (Folkman and Klagsbrun, 1987).

These facts appear to make the analyses of the structure and function specific to bFGF very complicated, and the development of MoAbs against the bFGF molecule are urgently required.

In this chapter, the development of MoAbs against the mature form of human bFGF as specific probes is described. Isolated MoAbs identified the mature and the truncated form of human bFGF and could distinguish bFGF from aFGF.

6-2. MONOCLONAL ANTIBODIES AGAINST HUMAN BASIC FGF

6-2-1. Human bFGF MoAbs produced by hybridoma cells

Four murine hybridomas secreting an antibody to human bFGF were selected from an original pool of hybridoma cultures using an EIA procedure. The antibodies secreted by these hybridomas were designated as MoAb12, MoAb52, MoAb78, and MoAb98. Their antibody isotypes were IgG_1 for MoAb12 and MoAb98 and IgG_{2b} for MoAb52 and MoAb78. These four IgG-type antibodies were produced in ascites. Through limited dilution, these ascitic fluids showed

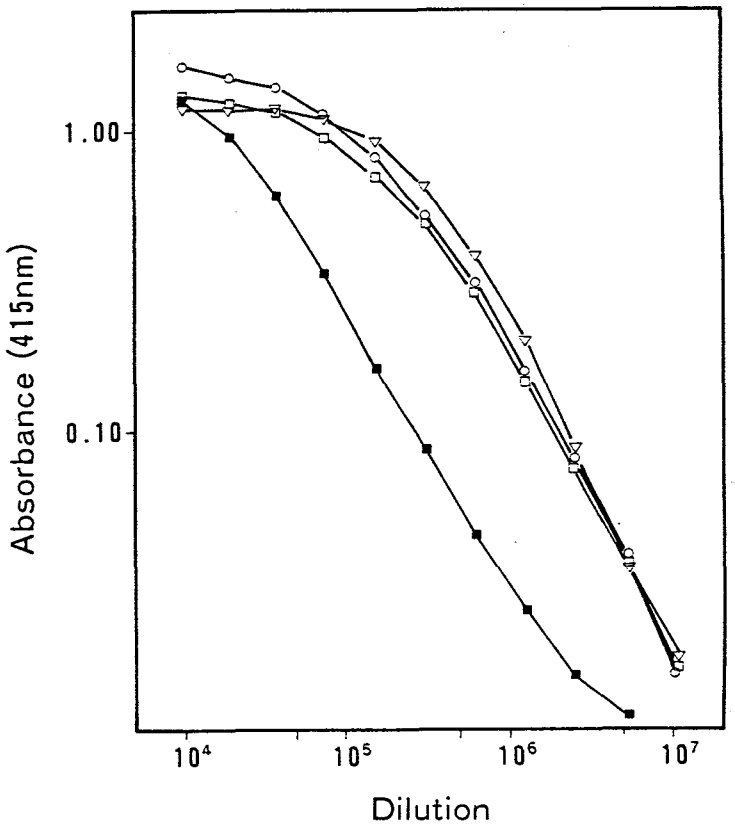

Figure 6-1. Binding ability of MoAbs to human bFGF. Ascites fluid collected from mice injected with a hybridoma producing an IgG type MoAb was used in the EIA assay as described in [3-5-3]. Each fluid was sequencially diluted and bound to solid-phase human bFGF. The HRP reaction measured at 415nm qualified the binding ability to human bFGF of MoAb12 (○), MoAb52 (□), MoAb78 (■), and MoAb98 (▽).

affinity for human bFGF at dilutions of up to 10^{-6} in the case of MoAb78 and 10^{-7} in the case of MoAb12, MoAb52, and MoAb98 (Fig.6-1).

6-2-2 Amino-terminal truncated human bFGF

To mimic the human bFGF lacking the 15 amino-terminal amino acid residues, the truncated form found in several tissues (Gospodarowicz et al., 1987), plasmid pTB795 was constructed using site directed mutagenesis (Fig.3-2) to produce N14, human bFGF lacking the first 13 amino acid residues, in E.coli. Simultaneously, plasmid pTB855 for the expression of N41, human bFGF lacking 40 amino acids at its amino terminal, was constructed (Fig.3-2) by inserting the ATG codon for the initiation of translation at the restriction site of HincII present in the human bFGF cDNA (Kurokawa et al., 1987). Both N14 and N41 were purified from E.coli cells containing plasmids pTB795 and pTB855 by the procedure using heparin affinity column chromatography described for bFGF [Chapter 4] (Fig.6-2). Growth stimulation of BALB/c3T3 cells by N14 and N41 was assayed by monitoring the incorporation of [^{3}H]-thymidine in DNA synthesis. N14 stimulated the growth of BALB/c3T3 cells as well as the mature form of human bFGF. N41 was less active than human bFGF but was not inactive. The specific activity of N41 purified by heparin affinity column chromatography was approximately 1/100 of that of the mature form of human bFGF (See "Chapter 7" for details.)

6-2-3. Identification of the epitopes defined by the MoAbs

To determine whether the four MoAbs bound to similar or to different epitopes on human bFGF, competition on EIA was examined by employing synthetic polypeptides Pep1 (a replicate of the

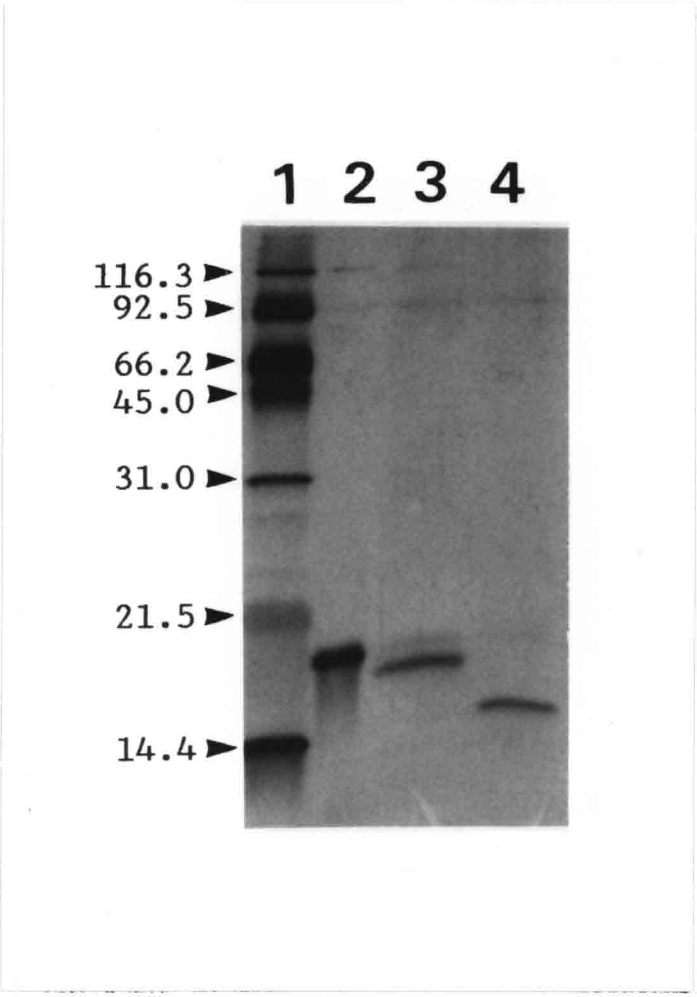

Figure 6-2. Amino-terminal truncated human bFGFs. N14 and N41 purified by heparin affinity column were analyzed in 17.25% SDS-PAGE stained with silver. Lanes:1, Molecular weight markers (Units were in kilodaltons.); 2, human bFGF purified from E.coli harboring plasmid pTB669; 3, N14 purified from E.coli harboring plasmid pTB795; 4,N41 purified from E.coli harboring plasmid pTB855.

amino-terminal of human bFGF, amino acid 1-9) and Pep3 (a replicate of the carboxy-terminal of human bFGF, amino acid 141-146), and amino-terminal truncated human bFGFs, N14 and N41, described above as competitors. As shown in Fig.6-3, Pep1 prevented MoAbs12 and MoAb78 from binding to human bFGF, while neither synthetic polypeptide prevented MoAb52 or MoAb98 from binding (Fig.6-3). Human bFGF competed with N14 in binding to MoAb52 and MoAb98, but did not competed with N41 (Fig.6-4). Thus at least two distinct epitopes were identified, one recognized by MoAb12 and MoAb78 and the other by MoAb52 and MoAb98. From these results, it is evident that MoAb12 and MoAb78 recognize the epitope found only in the mature form of human bFGF. A probable location for that epitope would be on the amino-terminal region (1-9 amino acid) of the mature form of human bFGF. Alternatively MoAb52 and MoAb98 recognized the region amino acid 14-40 which was present in N14 but was not in N41.

6-2-4. Binding specificity of MoAbs to human bFGF

The specificity of the four MoAbs to human bFGF was determined by EIA using 10ug/ml of human bFGF and bovine aFGF as competitors. Even in the presence of bovine aFGF, it was possible to detect human bFGF bound to the plate quantitatively, while in the presence of human bFGF the absorbance at 415nm was reduced to less than 40% of the control without any competitors (Fig.6-5).

6-2-5. Sandwich EIA for human bFGF

A sandwich EIA was used to evaluate the ability of the MoAbs to quantitate the mature form of human bFGF. First, solid-phase antibodies and solution-phase enzyme labeled antibodies were

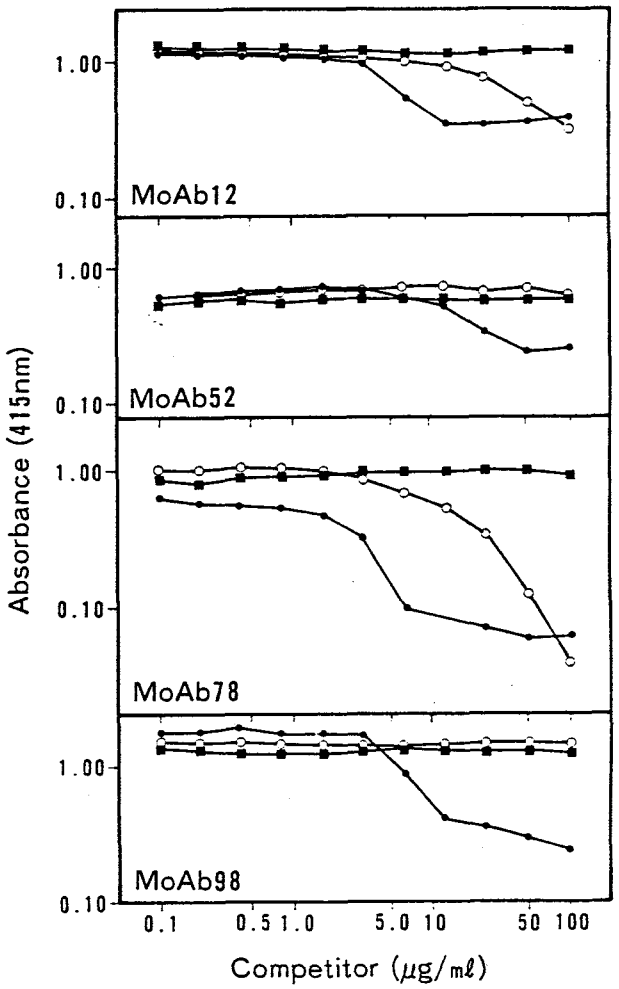

Figure 6-3. Competition of human bFGF with synthetic polypeptides in binding to 4 MoAbs. The competitors Pepl (○) , Pep3 (■), and human bFGF (●) were added at concentrations ranging from 0.lug/ml to 100ug/ml; the HRP reaction was monitored at 415nm.

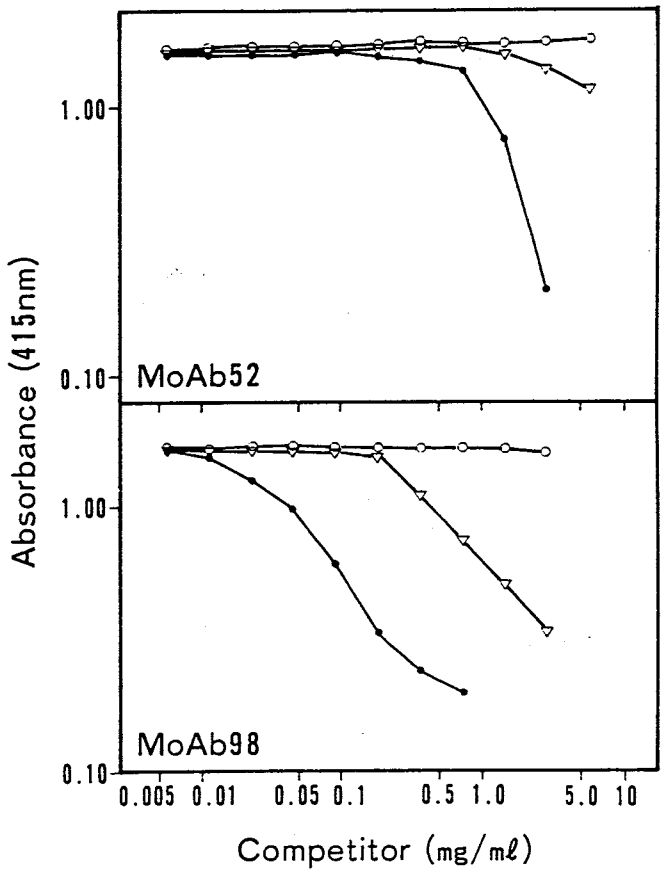

Figure 6-4. Competition of human bFGF with truncated human bFGF in binding to MoAb52 and MoAB98. The bacterial crude extracts that contained human bFGF (●), N14 (▽), and N41 (○) were added as competitors at concentrations ranging from 0.005mg/ml to 5mg/ml of total protein; HRP reaction was monitored at 415nm.

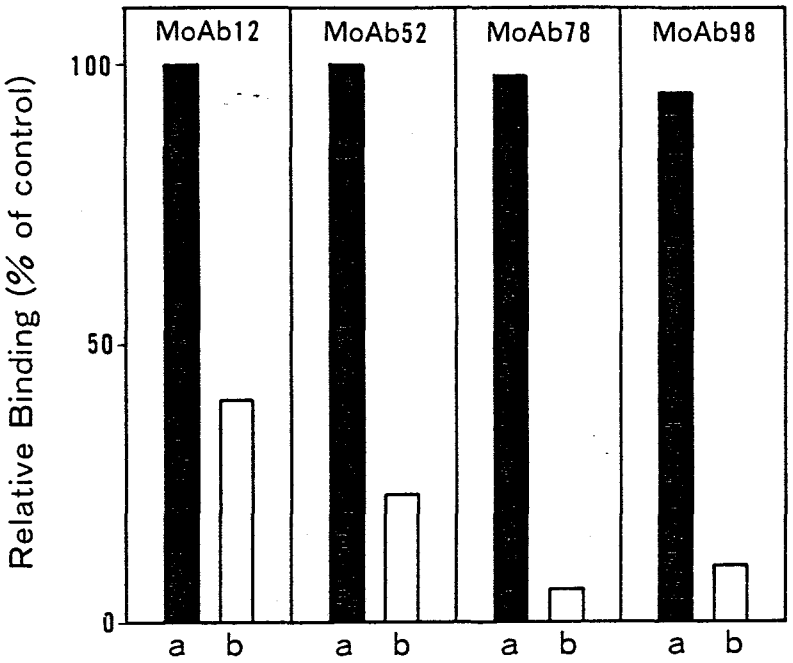

Figure 6-5. Binding specificity of MoAbs for human bFGF. Binding of IgG type-MoAbs (MoAb12, MoAb52, MoAb78, and MoAb98) to human bFGF influenced by 10ug/ml of bovine aFGF (a) or human bFGF (b) was assayed in an EIA system as described in [3-5-3]. Relative binding is calculated as the % of control (absorbance for human bFGF without any competitors).

tested in various combinations to detect the mature form of human bFGF. The optimum combination resulted when solid-phase attached MoAb52 (2ug/well) or MoAb98 (1ug/well) and soluble enzyme-labeled MoAb78 were used. The detection of increasing concentrations of human bFGF (ranging from 0.1 ng/ml to 50 ng/ml) is shown in Fig.6-6. In both cases, the EIA is sensitive enough to detect 0.5 ng/ml of human bFGF. Thus this sandwich EIA can be used to measure specifically the mature form of human bFGF.

6-2-6. Purification of human bFGF mutein CS4 by MoAb78

Attempts were made to purify the human bFGF mutein CS4 [Chapter 5]. A crude extract of bacterial cells harboring plasmid pTB744 (Table 5-1) was applied to the column. Almost all the human bFGF bound to the column. The column was washed with buffer containing 1M NaCl, and then the protein was eluted with two different buffers, one at pH4.5 and one at pH2.5, as shown in Fig.6-7A. Little activity was detected in the fraction washed out and that eluted at pH4.5. Only the fraction eluted at pH2.5 caused the accelerated incorporation of ^{3}H-thymidine in the DNA synthesis of BALB/c3T3 cells. The bacterial crude extract and each fraction, including the one which passed through the column, were analyzed on SDS-PAGE (Fig.6-7B). The main band of protein migrating at the expected MW of 17K daltons was recognized in only the fraction eluted at pH2.5, which showed specific activity almost equal to that of human bFGF as is shown in Table 5-1.

6-3. DISCUSSION

MoAbs to human bFGF were prepared to detect specific epitopes, determine the immunologic cross reactivity of different amino-terminal truncated human bFGF, develop an immunoassay to

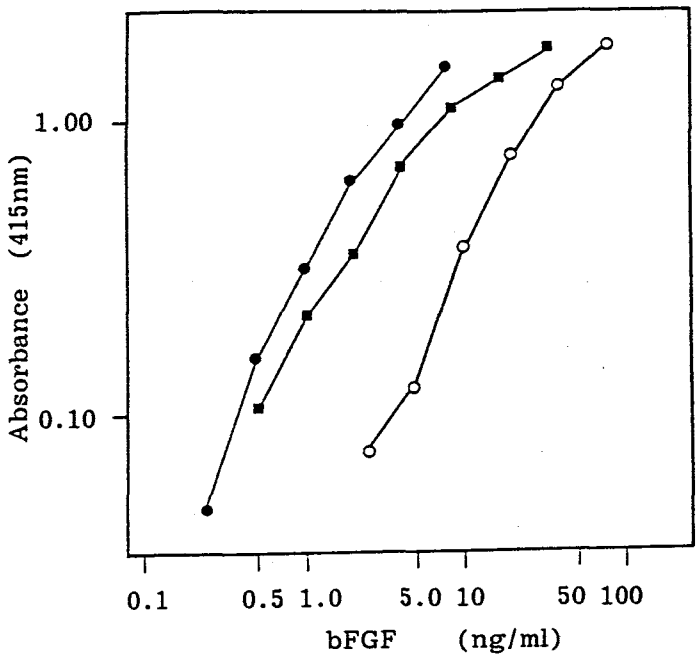

Figure 6-6. Sandwich EIA for human bFGF. Two pairs of MoAbs were used in the sandwich EIA. One is the pair MoAb52 (○,1ug/well; ●,2ug/well) in solid phase and MoAb78 conjugated with HRP in solution phase. The other pair is MoAb98 (■,1ug/well) in solid phase and MoAb78 in solution phase. HRP reaction was monitored at 415nm.

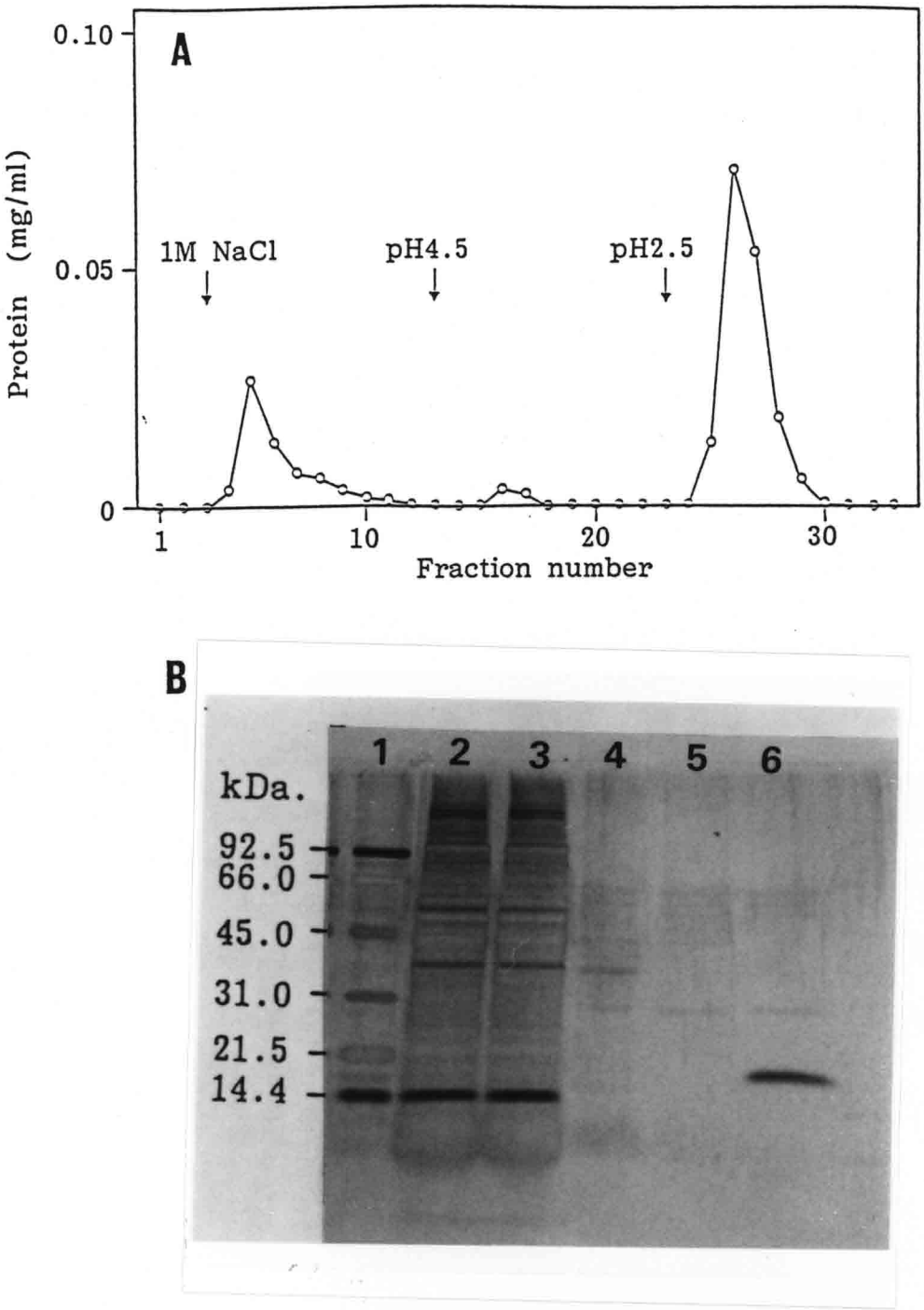

Figure 6-7. Purification of modified bFGF, CS4, using an MoAb78-affinity column. (A) Bacterial crude extract containing human bFGF mutein CS4 was applied to an MoAB78 affinity column, and the protein bound to the MoAb78-affinity column was eluted at pH4.5 and at pH2.5 after washing with neutral buffer containing 1M NaCl. The amount of protein in each fraction was assayed using a Protein-Assay kit (Bio-Rad). (B) The peak fractions obtained from the profile above were analyzed using SDS-PAGE and silver staining. Lanes: 1, molecular weight marker; 2, crude extract; 3, fraction which passed through the column; 4, fraction washed out of the column; 5, fraction eluted at pH4.5; 6, fraction eluted at pH2.5. In lane 2 and 3, 10ug of total protein was applied, and 100ng of total protein was applied in lanes 4-6. Units are in kilodaltons.

distinguish bFGF from aFGF and other growth factors, and to purify a human bFGF mutein that was not purified efficiently using a heparin affinity column. There were several technical problems in the production of the monoclonal antibodies due to the unique properties of bFGF. This growth factor was found to be a poor immunogen, reflecting its highly conserved amino acid sequence (Abraham et al., 1986a,b; Kurokawa et al., 1987). Massoglia reported four monoclonal antibodies to bovine pituitary bFGF (Massoglia et al., 1987). Three of their antibodies also recognized the amino-terminal region of bFGF (amino acid 1-10). From our results reported here and taking theirs into consideration, the amino-terminal sequence of bFGF seems to be externally exposed in the molecule and seems to be suitable as an antigen.

The 4 MoAbs recognize two distinct epitopes at the amino terminal portion of human bFGF. Two of them (MoAb12 and MoAb78) recognize the epitope(s) located within the first 9 residues of the mature form of human bFGF. This epitope seems to be specific to bFGF when compared with the primary structure of aFGF. The other two antibodies (MoAb52 and MoAb98) recognize the epitope(s) within the amino-terminal side of the core of human bFGF (amino acid 14-40), where a 60% homology with aFGF exists, while the total homology between bFGF and aFGF is 55% (Esch et al., 1985b). In spite of this fact, the EIAs using MoAb52 and MoAb98 were not influenced by aFGF (Fig.6-5). MoAb52 binds very weakly to N14 (Fig.6-1). This implies that MoAb52 recognizes a site around the 14th amino acid residue from the amino-terminal of the mature form of human bFGF. N14 inhibited MoAb98 from binding to the mature form of human bFGF while N41 did not. These results indicate that MoAb98 recognizes a site between the 14th and 40th

amino acid of the mature form.

Considerable evidence already exists that the amino-terminal portion of bovine bFGF (amino acid 1-15) is not required for mitogenic activity or receptor binding (Gospodarowicz et al., 1985b, Neufeld and Gospodarowicz, 1985, 1986); this appears to be true for human bFGF as demonstrated in this paper by N14. The epitope recognized by MoAb52 and MoAb98 is apparently not intimately involved in mitogenic or receptor binding activity since these antibodies did not block the accelerated incorporation of ^{3}H-thymidine into DNA in quiescent BALB/c3T3 cells treated with human bFGF (data not shown). In spite of the speculation regarding the active domain (Baird et al., 1986), our results imply that N41 still has the domain for cellular growth stimulating activity. Other FGF assays, such as the cellular proliferation assay, do not distinguish the effects of aFGF and bFGF as both forms interact with the same cell surface receptor (Neufeld and Gospodarowicz, 1986). The 4 MoAbs described above have strict specificity for epitopes only on bFGF. This characteristic enables the precise measurement of bFGF without interference by aFGF.

MoAbs52, MoAb78, and MoAb98 have been used in a sandwich EIA specific for the mature form of human bFGF (amino acid 1-146). This EIA can quantitate bFGF even at a concentration of 0.5ng/ml, which is sensitive enough to detect tissue-produced human bFGF. Because many types of carcinoma cells producing FGF, such as chondrosarcoma, melanoma, and hepatoma (Shing et al., 1984; Klagsbrun et al., 1986; Lobb et al., 1986), exist, the sandwich EIA might be suitable for clinical use to survey the existence of cancer in patients.

Modified human bFGF CS4, which could not be purified efficiently using a heparin affinity column, was purified using MoAb78-affinity comlumn chromatography. CS4 was eluted from the column at pH2.5, which was low enough to inactivate bFGF (Westall et al., 1983); however, CS4 showed specific activity equal to that of human bFGF as described in chapter 5. The temperature of 4C, at which the chromatography was carried out, and the quick neutralization of the fraction would protect this mutein from inactivation. This finding facilitates the study of modified bFGF molecules which are also expected to be used clinically, because the possibilty of the effective creation of a bFGF molecule with less affinity to heparin by recent protein engineering cannot be denied, although heparin binding was reported to protect bFGF from inactivation (Gospodarowicz and Cheng, 1986).

Thus these results clearly demonstrate that the MoAbs described here enable small amounts of human bFGF to be detected; this will help in cancer surveillance. In addition these MoAbs are useful in the purification of human bFGF molecules modified by recent protein engineering, which are expected to be novel clinical drugs in areas such as wound healing.

CHAPTER 7

STRUCTURAL ANALYSIS OF THE HEPARIN-BINDING DOMAIN OF BASIC FGF

7-1. INTRODUCTION

Extraordinarily high affinity for heparin is one of the important characteristics of the FGF family. In fact, this characteristic has been applied to the isolation and characterization of the proteins in this family, especially bFGF and aFGF, from various tissues (Baird et al., 1986) and tumors (Shing et al., 1984). The identification of fundamental domains of bFGF specific for receptor- and heparin-binding has been reported using synthetic polypeptides; these have implied the existence of two domains for heparin-binding and one for receptor-binding (Baird et al., 1986, 1988).

We constructed several bFGF molecules whose amino- or carboxyl-terminus was deleted. The structural characterization for heparin-binding is described in this chapter using these terminal truncated molecules.

7-2. AFFINITY FOR HEPARIN AND TERMINAL TRUNCATED FGFS

7-2-1. Comparison of the isoelectric point indexes in the FGF family

The pI value of each amino acid composing the proteins was plotted along with its position in the amino acid sequence (Fig.7-1). This process was applied to the FGF family, which consists of several proteins, namely the amino acid sequences of human bFGF (Abraham et al., 1986b; Kurokawa et al., 1987), human aFGF (Jaye et al., 1986), int-2 (Moore et al., 1986), hst (Yoshida et al., 1987)/ KS3 (Delli-Bovi et al., 1987), and FGF-5

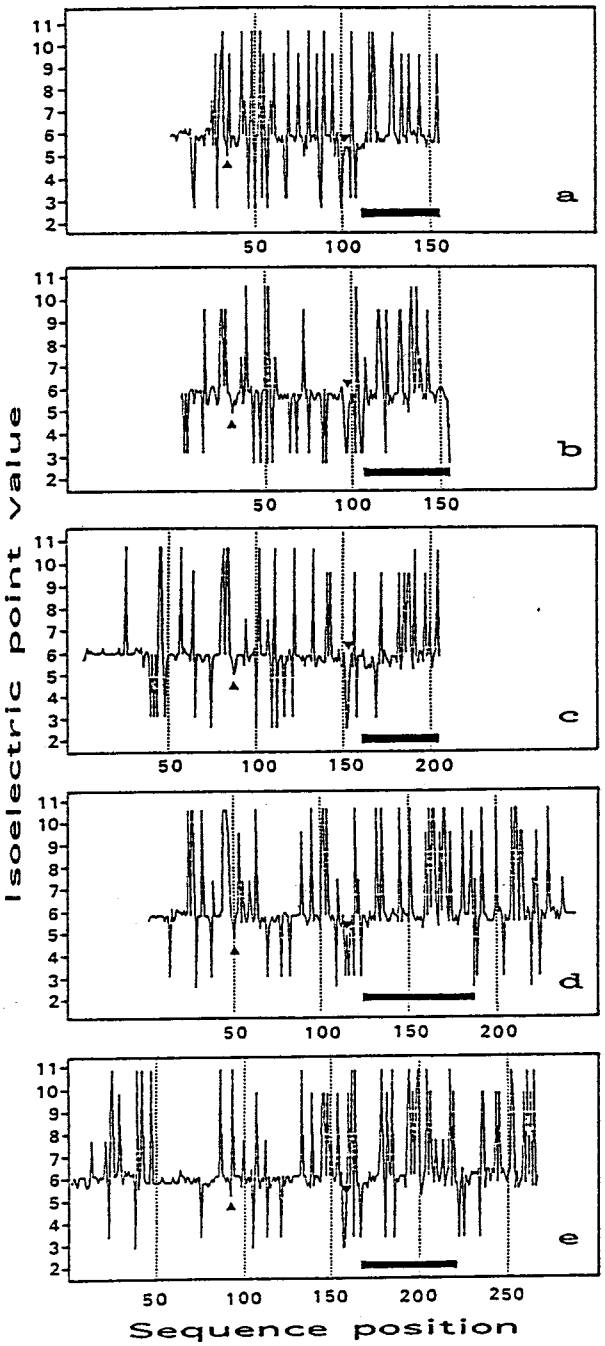

Figure 7-1. Isoelectric point indexes of the FGF family. The pI value of each amino acid composing a protein is plotted according to its sequence position. (a) human bFGF (155 amino acids including 9 amino acids of signal like sequence). (b) human aFGF (155 amino acids including 15 amino acids of signal like sequence). (c) hst-1/KS3 (206 amino acids). (d) int-2 (240 amino acids). (e) FGF-5 (267 amino acids). The horizontal solid bars show the basic region located in the carboxyl-terminal of human bFGF and each corresponding region in the other members of the FGF family when alignment for homology is made. The positions of the cysteine residues conserved in FGF family are marked. (▲) is at the position of Cys^{25} and (▼) at Cys^{92} in mature bFGF. For comparison purposes the position of Cys^{92} is set at the same position for each FGF molecule.

(Zhan et al., 1988). Though the mature forms of bFGF and aFGF consist of 146 and 140 amino acid residues, respectively, the signal-like sequences present in both bFGF and aFGF are included in the sequence position, and their length is shown as 155 amino acid residues. As shown in Fig.7-1 the carboxyl-terminus of bFGF, from Asn^{101} to Ser^{146} (underlined in Fig.7-2 A), is extremely rich in basic amino acid residues and has no acidic residues, and the relatively homologous region of each member protein in the family as described previously (Dickson and Peters, 1987; Zhan et al., 1988) is shown by a solid horizontal bar in Fig.7-1. In this region of each member, there is an abundance of basic amino acid residues, but few acidic ones are found. The alignment, setting one of the two cysteine residues (Cys^{92} in bFGF) completely conserved in the family at the same position, enables us to show the position of this region as well as the other cysteine residue (Cys^{25} in bFGF) at the same position (Fig.7-1).

7-2-2. Terminal truncated bFGF molecules

Using a recombinant DNA technique, 12 different terminal truncated bFGF molecules were produced in E.coli: two amino-terminal truncated molecules, N14 and N41, and ten carboxyl-terminal truncated molecules, C100, C104, C113, C117, C122, C128, C137, C141, C143, and C144. The procedure used to construct the cDNA coding for amino-terminal truncated bFGF was as described [3-9-1]. To construct the one coding the carboxyl-terminal truncated bFGF, the bFGF cDNA coding the mature bFGF, which consists of 146 amino acids (Fig.7-2 A), was treated with Exonuclease III coupled with Mung-bean nuclease as shown in Fig.3-3. A linker having a nonsense codon in each frame was

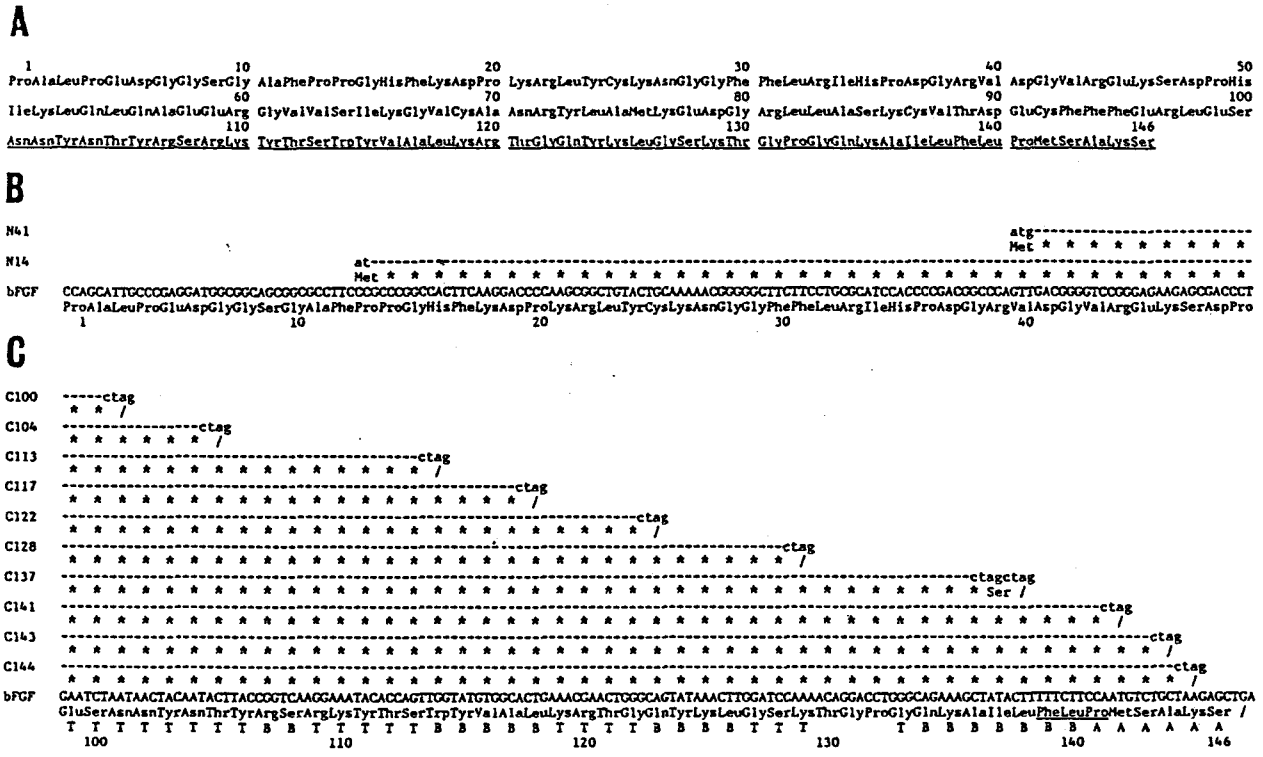

Figure 7-2. The structure of mature human bFGF (A), amino-terminal structures of amino-terminal truncated bFGFs (B), and carboxyl-terminal structures of carboxyl-terminal truncated bFGFs (C). A, primary structure of human bFGF composed of 146 amino acids. B, C, the nucleotide sequences and deduced amino acid sequences at the terminal are shown. Each nucleotide identical to human bFGF is depicted by "-," and each amino acid identical to human bFGF is depicted by "*." Nucleotides in lower case are derived from the modification or linker for translational initiation codon "ATG" (B) and the nonsense codon linker (5'-CTAGCTAGCTAG-3') (C). Each "/" shows the position of an effective stop codon (C). The underlined amino acid sequences are the basic region shown in Fig.7-1 a (A) and the sequence present in all members of the FGF family discussed in the text (C). The secondary structure predicted from the amino acid sequence is shown on the bottom line. "T" depicts beta-turn, "A" alpha-helix, "B" beta-sheet, and blank random coil (C).

ligated to the deleted end of the cDNA to encode carboxyl-terminal truncated bFGFs. The cDNAs having nonsense codons at the appropriate portions were screened by nucleotide sequencing to obtain the 10 molecules.

In detail for the terminal sequences, N14 lacks 13 amino acid residues in the amino-terminal, Pro^{1}-Pro^{13}, replacing Pro^{13} with Met, and N41 lacks 40, Pro^{1}-Val^{40}, putting the ATG codon before the codon for Asp^{41} as described [3-9-1] (Fig.7-2 B). C100 has Ser^{100} in its carboxyl-terminal; C104, Asn^{104}; C113, Ser^{113}; C117, Ala^{117}; C122, Gly^{122}; C128, Ser^{128}; C141, Pro^{141}; C143, Ser^{143}; C144, Ala^{144}; and C137 has an additional Ser^{137} residue (Fig.7-2 C). As shown in Fig.7-3 a and b, the molecules C141, C143, and C144 were efficiently purified by the procedure used for the purification of recombinant human bFGF [3-1] as well as N14 and N41 (Fig.6-2). Although they were produced in sufficient amounts without degradation as shown in Fig.7-3 c and d, the other 7 molecules could not be purified efficiently because they could not be easily extracted from E.coli. However, a small amount of solubilized molecules were detected upon the two-site EIA using monoclonal antibodies [3-6-2].

7-2-3. Elution profiles of carboxyl-terminal truncated bFGF in heparin affinity HPLC

The affinity of each carboxyl-terminal truncated bFGF molecule for heparin was examined using a heparin affinity HPLC procedure [3-1]. Each sample was applied to the column in the presence of 5mM DTT to avoid multimer formation by disulfied bonding [Chapter 4 and 5] and was eluted under a concentration gradient of NaCl, from 0 to 2M. Each truncated bFGF protein in fractions eluted from the column was monitored and quantified by a two-site EIA.

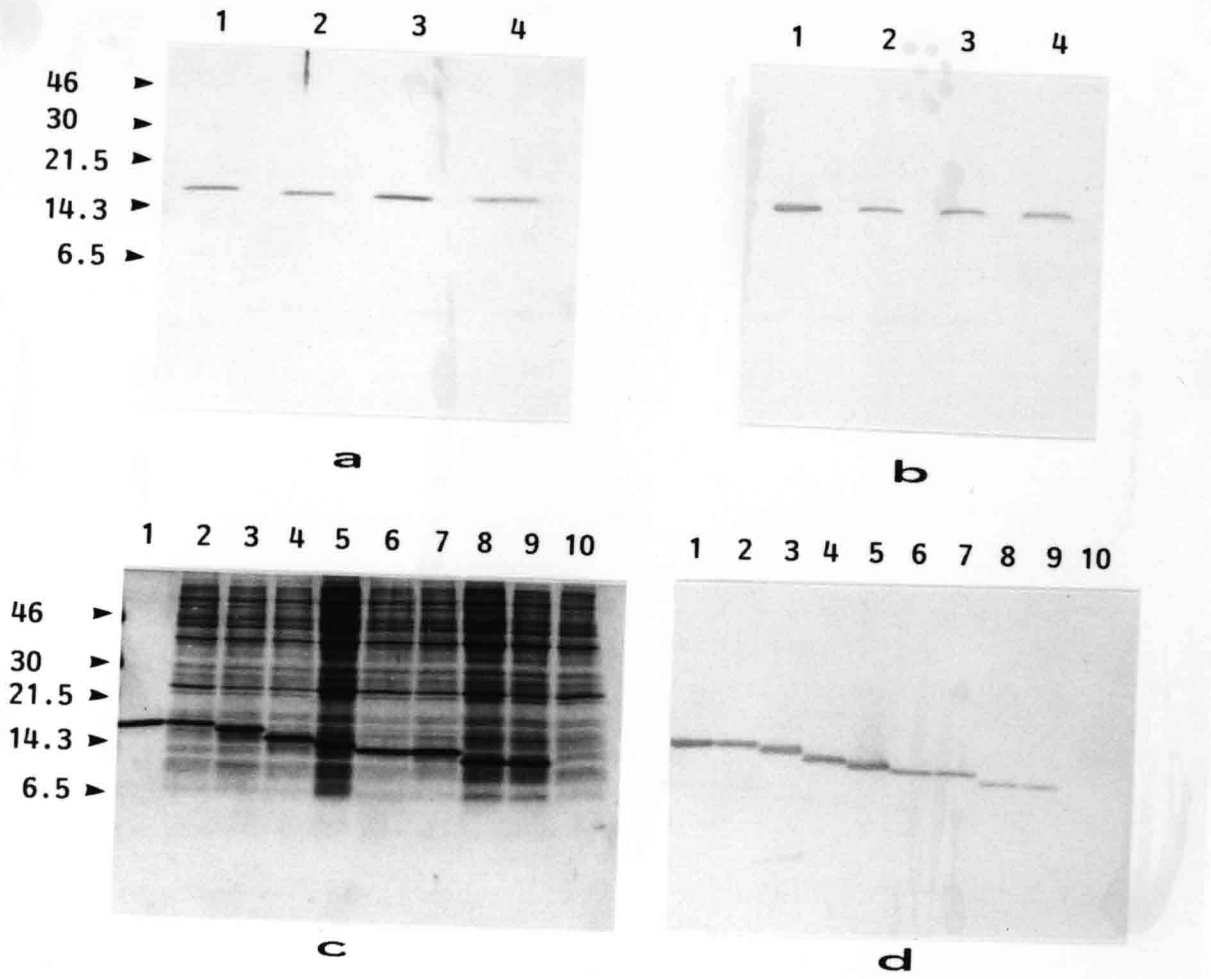

Figure 7-3. Identification of carboxyl-terminal truncated bFGFs. Coomassie Blue-stained 20% SDS PAGE gels (a,c) and Western blotting analyses with anti human bFGF monoclonal antibody MAb78 (b,d). (a,b), samples purified using heparin HPLC, lane 1, recombinant human bFGF; lane 2, C144; lane 3, C143; lane 4, C141. (c,d), lane 1, purified recombinant human bFGF as a positive control; lane 2-9, whole bacterial lysates, samples are bFGF (lane 2), C137 (lane 3), C128 (lane 4), C122 (lane 5), C117 (lane 6), C113 (lane 7), C104 (lane 8), C100 (lane 9) and whole cell lysate of bacteria harboring the plasmid pET3c as a negative control (lane 10). The horizontal arrows with molecular weight values ($MWx10^{-3}$) indicate the position of molecular weight markers.

Their elution profiles are shown in Fig.7-4. Truncated bFGFs were classified into 3 classes. One exhibited a sharp peak eluted at the concentration of 1.3M NaCl, which is identical to the ordinary profiles of mature bFGF, N14 and N41 (Fig.7-4 b, c, d); another exhibited a single sharp peak at the concentration of 0.3M NaCl (Fig.7-4 j, k), which indicates a weak affinity for heparin; and the other exhibited two or three peaks between the concentrations of 0.3 to 1.3M NaCl (Fig.7-4 e, f, g, h, i). Drastic changes occurred in the elution profiles of C137 and C141, which implies that the sequence, $Ile^{137}Leu^{138}Phe^{139}Leu^{140}Pro^{141}$, is essential for the bFGF molecule to maintain a stable conformation. In accordance with the truncation of more than 10 amino acid residues from the carboxyl-terminal, the profiles of the heparin HPLC began to show a pattern of perturbation, which finally fell into one peak recognized in the profile of C104. This perturbation was not observed in the profiles of C104 or C100 lacking the basic region.

7-2-4. Activity of terminal truncated bFGF molecules

The biological activity of each terminal truncated bFGF fractionated through the heparin affinity HPLC column to stimulate the DNA synthesis in BALB/c3T3 cells was examined. As shown in Table 7-1 the activity of each peak fraction was calculated in relation to the ratio of the molecular weight of the terminal truncated bFGF to that of the mature form: the molecular weights were calculated theoretically according to the amino acid components predicted from the cDNA sequence. The carboxyl-terminal truncated bFGF molecules singularly eluted with 1.3M NaCl, C141, C143, and C144, showed activity almost equal to

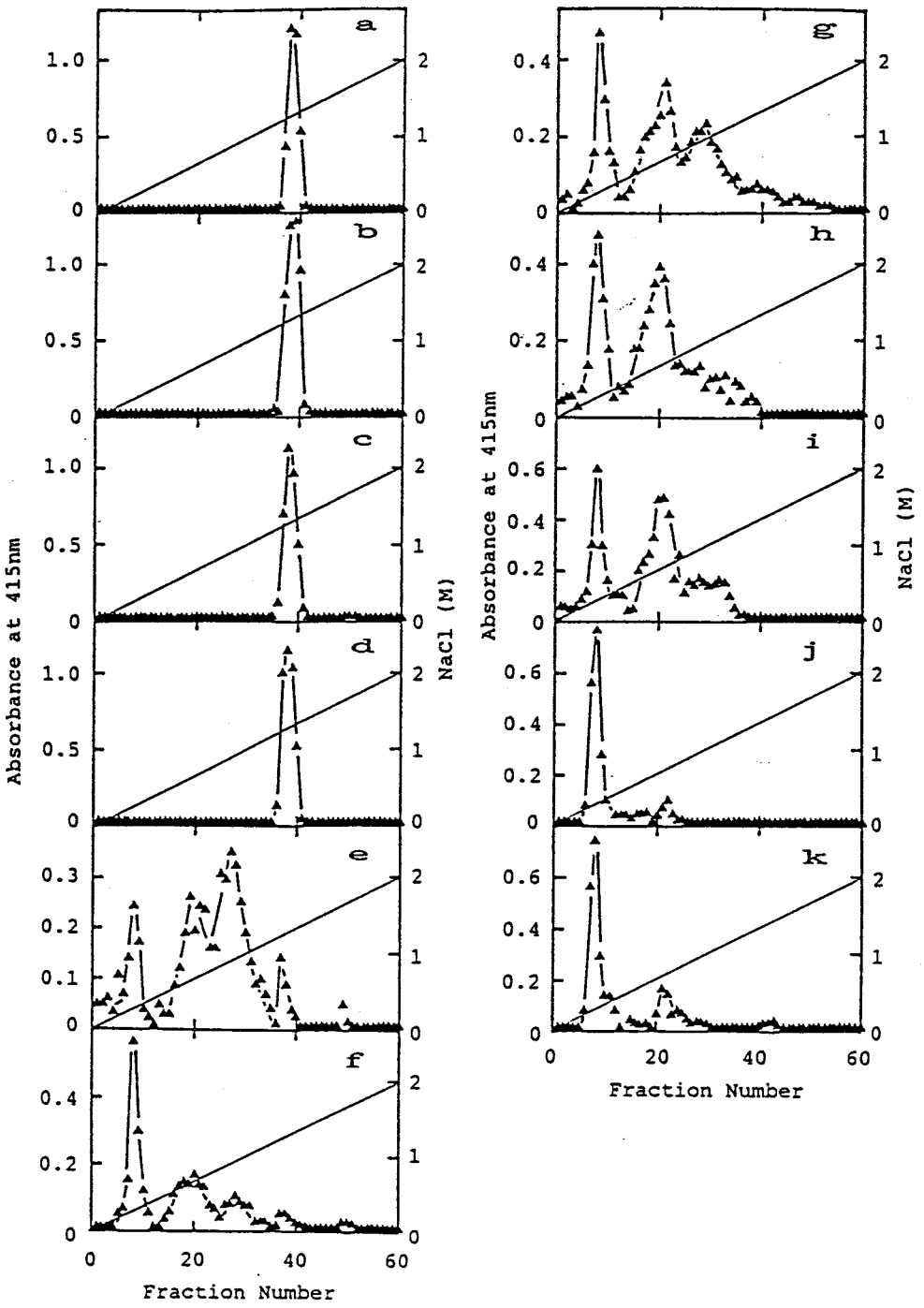

Figure 7-4. Elution profiles of heparin affinity HPLC of carboxyl-terminal truncated bFGFs. Purified human bFGF (a), C144 (b), C143(c), and C141(d), and bacterial extracts of C137 (e), C128 (f), C122 (g), C117 (h), C113 (i), C104 (j), and C100 (k) were applied to a heparin affinity HPLC column and eluted under a concentration gradient of NaCl (0-2M). Eluted fractions were assayed using a 2-site EIA, and absorbance at 415nm was monitored (▲).

that of the mature molecule. The further truncated molecules, C113, C117, C122, C128, and C137 exhibiting several peaks at concentrations of NaCl between 0.3 and 1.0M which implied a weakened affinity for heparin, showed activity of 0.1% to 35% when the activity of mature bFGF was assumed to be 100% (Table 7-1). Eluted with 0.3M NaCl, C100 and C104 lacking almost the entire basic region at the carboxyl-terminal of bFGF were not inactive but showed activity significantly less than 0.1% that of mature bFGF (Table 7-1). The activity of the amino-terminal truncated molecules, N14 and N41, purified as described in chapter 6 was also determined. N14 is a mimic of bFGF purified from kidney and corpus luteum (Esch et al., 1985a; Gospodarowicz et al., 1985a) and showed an activity of 68% that of the mature form of bFGF. Furthermore, N41 showed activity of about 2.3%, which is stronger than that of C100 or C104 (Table 7-1).

7-3. DISCUSSION

The strong affinity for heparin is one of the most prominent characteristics of FGF. This affinity is considered to be involved in binding to cell surface matrixes which is distinguishable from receptor binding, so called high affinity binding (Moscatelli, 1988). The physiological role of FGF's affinity for heparin is not clear, but it is considered to potentiate and stabilize the molecule (Schreiber et al., 1985) by protecting it from inactivation (Gospodarowicz and Cheng, 1986) or proteolytic degradation (Saksela et al., 1988). We thought this affinity would be derived from ionic charge, because extracellular matrixes such as heparin are usually negatively charged, and bFGF, whose pI is 9.6 (Gospodarowicz et al., 1985a, 1985b), has a strong positive charge. The pI index of bFGF

Table 7-1. MOLECULAR WEIGHTS AND BIOLOGICAL ACTIVITY OF TERMINAL TRUNCATED BASIC FGFS

Molecular weight (MW) of each molecule was theoretically calculated from the amino acid residues deduced from the nucleotide sequence. Affinity for heparin was defined by the NaCl concentration at which each peak in the heparin affinity HPLC profiles occurred (Fig.7-4). The concentration of carboxyl-terminal truncated bFGF in each peak fraction was determined by a 2-site EIA, and the concentration of amino-terminal truncated bFGF was determined by the absorbance at 280nm. The biological activity of each peak fraction is shown as a percentage with that of mature bFGF being 100.

Sample	$MWx10^{-3}$	Affinity for heparin (M)	Relative activity
C100	11.3	0.3	$1.7x10^{-2}$
C104	11.8	0.3	$0.7x10^{-2}$
C113	12.9	0.3	5.0
		0.7	20.0
C117	13.5	0.3	11.0
		0.7	8.0
C122	14.0	0.3	3.0
		0.7	8.0
		1.0	1.0
C128	14.7	0.3	15.0
		0.7	0.3
		1.0	0.1
C137	15.5	0.3	12.5
		0.7	3.0
		1.0	0.4
		1.3	34.8
C141	16.0	1.3	77.6
C143	16.2	1.3	135.2
C144	16.3	1.3	136.6
N14	15.3	1.3	67.9
N41	12.2	1.3	2.3
bFGF	16.5	1.3	100.0

(Fig.7-1) shows the presence of an extremely basic region at the carboxyl-terminus (around Asn^{101} to Ser^{146}), which is one of the domains of the molecule reported to be responsible for heparin binding (Baird et al., 1986, 1988). As shown by the solid horizontal bars in Fig.7-1, the basic character of the corresponding region is present throughout the entire FGF family taking the alignment along with the sequence homology (Dickson and Peters, 1987; Zhan et al., 1988) into consideration. Surprisingly this characteristic is not related to the pI of the proteins, for example the pI of aFGF is about 5 to 6 (Thomas et al., 1984).

To study the involvement of the carboxyl-terminal structure of bFGF in the molecule's heparin binding ability, we constructed carboxyl-terminal truncated bFGFs using a recombinant DNA technique utilizing Exonuclease III and Mung-bean nuclease as well as amino-terminal truncated bFGFs, N14 and N41 [3-9]. We obtained 10 carboxyl-terminal truncated bFGFs (Fig.7-2). In detail, C100 has Ser^{100} in its carboxyl-terminal; C104, Asn^{104}; C113, Ser^{113}; C117, Ala^{117}; C122, Gly^{122}; C128, Ser^{128}; C141, Pro^{141}; C143, Ser^{143}; C144, Ala^{144}; and C137 has an additional Ser^{137} residue at the carboxyl-terminus. These truncated molecules are divided into three groups. The first group completely lacks the basic region present at the carboxyl-terminus of bFGF including one of the "heparin-binding sites," $Arg^{107}Ser^{108}Arg^{109}Lys^{110}$, reported by Baird et al. (1986). The second has this "heparin binding site" but lacks $Phe^{139}Leu^{140}Pro^{141}$ which is present in all members of the FGF family. The last group has both of these two key sequences. The molecules in the first group showed a single peak eluted from

the heparin HPLC column at 0.3M NaCl (Fig.7-4) and still contained weak biological activity (Table 1). This indicates that the essential domain for receptor-binding has not been deleted completely. In addition, the fact that N41 and C100 can stimulate the DNA synthesis of BALB/c3T3 cells indicates that the essential domain for receptor-binding lies between Asp^{41} and Ser^{100}. The weak activity of the carboxyl-terminal truncated molecules may be a result of unstable conformation caused by deletion of the terminal sequence structure. Baird et al. (1986) reported two sequences, $Lys^{18}Asp^{19}Pro^{20}Lys^{21}Arg^{22}$ and $Arg^{107}Ser^{108}Arg^{109}Lys^{110}$, in mature bFGF concerned with the heparin-binding domain. Recently they pointed out that the latter one has higher affinity for heparin than the former one (Baird et al., 1988). These results are not consistent with ours in that N41, which lacks the former " heparin-binding site," was eluted at 1.3M NaCl from the heparin affinity HPLC column, and C100 and C104, which lack the latter "heparin-binding site," were eluted at 0.3M NaCl from the same column. Furthermore, taking into consideration the fact that the concentration of NaCl which elutes mature bFGF is the same as that which elutes N41, the affinity of the bFGF molecule for heparin does not appear to depend on the sequence $Lys^{18}Asp^{19}Pro^{20}Lys^{21}Arg^{22}$ but on the carboxyl-terminal sequence , from Thr^{105} to Pro^{141}, which is rich in basic amino acid residues, because the peaks at higher than 0.3M NaCl increased in proportion to the length of the carboxyl-terminal sequence which was present (Fig.7-4). Though it is not clear why these truncated bFGF molecules exhibited two or more distinct peaks, the multi-peak profiles in heparin affinity HPLC produced by C113, C117, C122, C128, and C137 might be the result of an unstable conformation caused by the absence of the terminal

sequence structure, and this disturbance does not seem to be observed in C100 and C104 exhibiting little affinity for heparin. This was not observed when the crude extracts of bFGF, C144, C143, and C141 were applied to the heparin HPLC column instead of using the purified proteins (data not shown). This fact eliminates the possibility of an effect of the bacterial proteins in the crude extracts on the multi-peak profiles. It is interesting to note that the longer the carboxyl-terminal sequence becomes, the higher the affinity for heparin becomes.

As is shown in Fig.7-3 and Fig.7-4, C141, C143, C144, and mature bFGF are easily extracted from E.coli and efficiently purified, while the other carboxyl-terminal truncated bFGFs produced in bacterial cells are extremely difficult to solubilize. We tried to solubilize these insoluble molecules more efficiently without inactivation. Detergents such as Triton-X100 and n-dodecyltrimethylammonium bromide and so on, and/or a higher concentration of NaCl (0.6 to 2M), were not effective. We also tried to culture the bacteria at 30C with mild induction of the promoter (Squires et al., 1988) to avoid the formation of inclusion bodies, and we tried to extract the molecules using an ion exchange resin (Hoess et al., 1988). But all of these methods have failed. This fact and the elution profiles of the carboxyl-terminal truncated bFGFs from the heparin affinity HPLC column indicate the existence of a structure between C137 and C141 which is involved in the stabilization of the bFGF molecule. The sequence, which C141 has and C137 does not, is $Ile^{137}Leu^{138}Phe^{139}Leu^{140}Pro^{141}$. Comparing the homology of the members of the FGF family (Dickson and Peters, 1987; Zhan et al., 1988), the sequence Phe-Leu-Pro is always present, and the

prediction of the secondary structure of the carboxyl-terminal sequence shows this sequence is located just between the predicted alpha-helix and beta-sheet (Fig.7-2). Absence of this sequence of three amino acid residues, which appears to break down the beta-sheet structure at the carboxyl-terminal, probably results in the insolubility of the molecules from E.coli which can be traced to the instability of the protein conformation.

Our study of the characteristics of the terminal-sequence structure presented here will lead to the development of molecules having modified heparin-binding activity, amplified biological activity, or antagonistic activity, because the multi-functional characteristics of bFGF suggest the possibility of a wide range of therapeutic applications.

CHAPTER 8

CONCLUSIONS

The conclusions drawn from the structural analyses of the human bFGF molecule described above are summarized as following:

[1] The cDNA for human bFGF was cloned from a human foreskin fibroblast cDNA library. The nucleotide sequence of the cDNA clarified the complete primary structure of this factor. Considering the partial amino acid sequence of bFGF purified from various tissues, the 9 amino acid residues at the amino-terminal of the protein are cleaved to give the mature form composed of 146 amino acid residues. To study the contribution of the four cysteine residues to the conformation of bFGF molecule, modified bFGFs having cysteine-to-serine changes were analyzed. The replacement Cys/25/Ser decreased the retention time of the main peak (p1) from the heparin HPLC column a little. In addition, the replacement Cys/25/Ser or Cys/92/Ser caused the peaks in the elution pattern to become rather dull and low. These observations indicate the loose binding of these types of modified bFGF molecules to heparin. However, decreased specific activity of p1 was only recognized with the replacement Cys/25/Ser. Three modified bFGFs (CS2, CS3, and CS23) showed the same biological activities as the original bFGF. CS23 showed a sharp single peak upon heparin HPLC even after being treated with an oxidizing reagent, but with CS2 and CS3, some protein was detected as heterologous peaks. These results indicate that the contribution of Cys^{69} and Cys^{87} to the conformation of the original bFGF molecule is reflected in the peaks of the elution profiles, that these cysteine residues are not

responsible for the affinity for heparin, and that they are exposed to the surface in the bFGF molecule allowing the formation of intra- or intermolecular sulfhydryl bridging, which results in several heterologous peaks upon heparin affinity chromatography.

[2] The involvement of the carboxyl-terminal structure of bFGF to the molecule's heparin binding ability was studied using amino- and carboxyl-terminal truncated bFGFs. The amino-terminal truncated bFGFs, N14 and N41, showed significant biological activity and complete heparin-binding activity. The carboxyl-terminal truncated molecules that completely lacked the basic region showed a single peak eluted from the heparin HPLC column at 0.3M NaCl and still contained weak biological activity. The peaks at higher than 0.3M NaCl increased in proportion to the length of the carboxyl-terminal sequence which was present. These results indicate that the essential domain for receptor-binding is located between Asp^{41} and Ser^{100} and that the affinity for heparin depends on the whole carboxyl-terminal sequence, from Asn^{101} to Ser^{146}, which is rich in basic amino acid residues. The elution profiles of the carboxyl-terminal truncated bFGFs upon heparin affinity HPLC indicate the existence of a critical sequence between Ile^{137} and Pro^{141} which stabilizes the bFGF molecule. The sequence Phe-Leu-Pro between Ile^{137} and Pro^{141} is always present in the FGF family, and it is implied that this sequence contributes to the stable structure of the bFGF molecule.

[3] The 4 MoAbs which we used recognized two distinct epitopes in the amino terminal of human bFGF. Two of them (MoAb12 and

MoAb78) recognized the epitope(s) located within the first 9 residues of the mature form of bFGF. The other two antibodies (MoAb52 and MoAb98) recognized the epitope(s) within the region Pro^{14} to Val^{40} in human bFGF. These MoAbs did not neutralize the mitogenic activity of bFGF. These results seem to indicate that the amino-terminal sequence of bFGF is externally exposed in the molecule and is not intimately involved in the active domain. This is consistent with the results obtained with the amino-terminal truncated molecule N41, which was still significantly active.

Modified bFGF molecules, including those obtained in these studies, will lead to the development of molecules resistant to anecedent oxiation and stabilized under acidic conditions which have modified heparin-binding activity, amplified biological activity, or antagonistic activity, since bFGF with its multi-functional characteristics suggests the possibility of a wide range of therapeutic applications such as accelerated remedies for injuries, burns and thrombi.

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