Summary

Objective: To investigate the toxic effect of cyclophosphamide (CPA) in the development of rodent molars.

Methods: CPA was administered intraperitoneally in postnatal mice between Day 1 and Day 10, and the morphological phenotype was evaluated at Day 26 using micro-computed tomography (CT) and histological analysis, including cell proliferation and cell death analyses.

Results: M3 molars of the mice who received 100 mg/kg CPA treatment at Day 6 or M2 molars who received treatment at Day 1 resulted in tooth agenesis or marked hypoplasia. Histological observation demonstrated that CPA treatment at Day 6 resulted in shrinkage of the M3 tooth germs, with significant reduction in the proliferation of apoptotic cells. Conversely, CPA exposure at Day 2, which occurs at around the bud stage of M3, resulted in crown and root hypoplasia, with reduced numbers of cusp and root. Additionally, CPA exposure at Day 10, which is the late bell stage of M3, induced root shortening; however, it did not affect crown morphogenesis.

Conclusion: Defective phenotypes were evident in both crown and roots due to the effect of CPA. Interestingly, the severity of the phenotypes was associated with the developmental stages of the tooth germs at the time of CPA administration. The

cap/early bell stage is the most susceptive timing for tooth agenesis, whereas the late bell stage is predominantly affected in terms of root formation by CPA administration.

Limitations: The timing of CPA administration is limited to after birth. Therefore, its effect during early stages of M1 and M2 could not be investigated.

Keywords: cyclophosphamide, hypoplasia, chemotherapy

Introduction

Recent advances in medical treatment for paediatric cancer have dramatically improved the survival rate after childhood cancer treatment (1,2). Presently, the 10-year survival rate of paediatric cancer patients of age <14 years is approximately 70% (1). As the survival rate of paediatric cancer patients has risen, the deterioration of the quality of life by the side-effects of cancer treatment has gained attention gradually.

Individual or combination of chemotherapy, radiotherapy and surgical approach are the common treatment options for current paediatric cancer patients. Chemotherapy and radiotherapy affect the development and growth of several organs in children (3). Furthermore, various complications such as endocrine disorders are late long-term effects (3,4). Conversely, in the oral cavity, dental anomalies, salivary gland dysfunction and craniofacial abnormalities occur as long-term side-effects of radiotherapy and

chemotherapy (5,6). Dental anomalies such as missing teeth, microdontia and shortening of the tooth roots are among the most common, long-term side-effects of radiotherapy and chemotherapy in the oral region that can cause malocclusion and affect facial development and impairment of health-related quality of life.

Cyclophosphamide (CPA) is one of the most successful drugs for paediatric cancer treatment. CPA is a prodrug of a derivative of nitrogen mustard that is metabolised by cytochrome P450 to produce phosphoramide mustard and acrolein (7). CPA-induced DNA damage is caused by DNA interstrand cross-links with phosphoramide mustard and oxidative stress with acrolein (7-9). Part of the damaged DNA is repaired normally by DNA repair mechanisms; however, the cells that cannot be repaired die due to apoptosis (7-12). Such cytotoxic action of CPA causes suppression of cell proliferation and cell death and enables the achievement of high reactivity to the cancer cells (7). Most paediatric cancer survivors receiving CPA treatment develop dental anomalies (5). Moreover, the phenotype and severity of dental anomalies differs with the timing, amounts and duration of CPA administration (13,14). These variations in dental anomalies can be attributed to most patients receiving CPA treatment when aged ≤5 years, and this timing is correlated with the developmental stage of each permanent teeth (5).

Tooth development is a complex process, wherein epithelial and mesenchymal interactions play a critical role and consist of multiple stages of tooth development. Tooth germs are formed in the stage order of bud, cap, early bell, late bell and root formation (15,16). The developmental stage varies for each tooth germ, and that of the second molar (M2) and the third molar (M3) are delayed by several days from the first molar (M1) in a neonatal mouse (17). Moreover, the distribution of proliferative cells changes according to the progression of the tooth developmental stage (18).

Previously, the effect of CPA administration on tooth development has been studied in rodents. It has been shown that undifferentiated cells in the pulp area were damaged after CPA administration in rats, resulting in dentin hypoplasia, pulp cavity narrowing and morphological abnormality of their incisors (19-22). Tooth root abnormalities, such as shortness, obliterated root and bone-like dentin formation, have been reported in rat M1 and M2 after CPA administration at the root formation stage (23-26). The degree of root abnormality becomes severe with increasing CPA concentration (25,26). Additionally, M3 became microdontia after CPA administration at the late bell stage in M3 (23,24). However, information on the effects of CPA on each tooth developmental stage is scare. Moreover, no study has demonstrated and elucidated the incidence and mechanism of the missing teeth induced by CPA administration in animal experiments.

Therefore, we examined the timing and dose-dependent effects of CPA administration on teeth development by morphological analysis using micro-CT. Additionally, histological analysis was performed to clarify the cellular mechanisms of dental anomalies induced by CPA administration.

Material and Methods

Laboratory animals

All animal experiments were performed in strict accordance with the guidelines of the Animal Care and Use Committee of the Osaka University Graduate School of Dentistry, Osaka, Japan. The Committee on the Ethics of Animal Experiments of the same university approved the study protocol (permit number: 29-033-0). Male and female C57BL/6 mice (Clea Japan, Tokyo, Japan) were used. K14-GFP mice were used to visualise the dental epithelial area during tooth development (27). All mice were housed with their mothers and provided *ad libitum* access to breast milk, a standard mouse feed and distilled water, and they were maintained under the conventional conditions of controlled temperature, humidity and a 12-h light–dark cycle.

Experimental design

The mice were divided into two groups: control and experimental. The experimental group received CPA via intraperitoneal (i.p.) injection (Endoxan, Wako Pure Chemical Industries, Ltd., Osaka, Japan) at a dosage of 25, 50 or 100 mg/kg. The dose of CPA was decided based on past studies on CPA concentrations used in acute myeloid leukaemia treatment (25,26). The control group received vehicle (PBS) injections of an equal volume prepared in phosphate-buffered saline (PBS). To investigate the effects of CPA treatment on tooth development at different stages and dosages and to examine the chronological changes after CPA treatment, 3 different experiment protocols were designed.

Experiment 1: Mice (n = 77) were randomly divided into 7 groups, which received either CPA or vehicle (control) at 1, 2, 4, 6, 8 and 10 days of age (Day 1, Day 2, Day 4, Day 6, Day 8 and Day 10, respectively). The experimental mice received CPA treatment at a dosage of 100 mg/kg body weight. After CPA administration, the mice were kept until 26 days of age, in which M3 erupted into the oral cavity (28). The number of surviving mice in all groups was counted as an index of systemic status. The mice who received injections of 100-CPA or vehicle at Day 6 were weighed at Day 6 and on every 2–4 days thereafter.

Experiment 2: Mice (n = 64) were used to study the dose-dependent effects of CPA on

tooth development. The mice were randomly divided into 3 groups and were administered CPA via i.p. injections at the dosages of 25, 50 and 100 mg/kg body weight or an equal volume of PBS at Day 4 or Day 6.

Experiment 3: Mice (n = 83) were used to study the chronological changes in mandibular molar development induced by CPA administration. For fluorescent staining of the sections, K14-GFP mice at Day 6 were used to visualise the dental epithelial area of the tooth germ. The mice were randomly divided into 2 groups and administered 100-CPA or vehicle at Day 6 via i.p. injections. The mice were randomly assigned to be euthanised on the day of injection (Day 6) or on 0.5 (Day 6.5), 1 (Day 7), 2 (Day 8), 4 (Day 10) or 6 (Day 12) days post injection.

Sample collection

At the end of the experiment, the mice from each group were anaesthetised with sodium pentobarbital (Nembutal, Dainihonseiyaku, Tokyo, Japan) and transcardially perfused with 4% paraformaldehyde. The mandible was dissected and fixed in 4% paraformaldehyde solution overnight at 4°C.

Three-dimensional (3D) analysis

For 3D visualisation and morphological evaluation of the mandibular molars, multiple

scans of the mice mandible were obtained by micro-CT (R_mCT2; Rigaku, Tokyo, Japan). The specimen image slices were recorded using 90-kVp tube voltage, 10-μm slice thickness and 160-μA tube current. An imaging software (Simple Viewer, viewer version 1.2.1, Rigaku, Tokyo, Japan) attached to micro-CT was used to perform computerised 3D reconstruction of the mandible.

Histological analysis

Histological changes in the mandibular molars and periodontal tissues were assessed by haematoxylin and eosin (HE) staining (29). Briefly, the fixed mandible of each mouse were decalcified with 10% EDTA at 4°C. To compare the positions and sizes of M2 and M3, the sagittal sections of 10-μm thickness were obtained via the conventional method. Then, the sections were stained with HE and observed under an optical microscope (BX 51TF; OLYMPUS, Tokyo, Japan).

BrdU labelling

Bromodeoxyuridine (BrdU) labelling was performed (29). The experimental mice were injected with BrdU (0.1 mg/g, ip), euthanised 2 h later and the sections were prepared as described above. Detection of BrdU was performed using the BrdU Staining Kit (Invitrogen Life Technologies, CA, USA) according to the manufacturer's instructions. Briefly, endogenous peroxidase was inactivated by treatment with 3% H_2O_2 in

methanol for 10 min, and the sections were incubated in trypsin and a denaturing solution for 10 and 25 min, respectively. All non-specific immunoreactivity was inhibited by treatment in the blocking solution for 10 min. Following incubation with biotinylated mouse anti-BrdU-antibody for 50 min, the sections were washed with PBS and reacted with streptavidin-peroxidase for 10 min. The signal was visualised using 3-3'-diaminobenzidine tetrahydrochloride (DAB) treatment for 5–10 min. After 3 distilled water washes, the immunostained sections were counterstained with haematoxylin.

Ki67 labelling

The sections were first incubated with the M.O.M. Mice Ig Blocking Reagent (Vector Laboratories, Inc., Burlingame, CA, USA) and then with primary antibodies in a diluent (1× PBS containing 8% protein concentrate; M.O.M.™ Kit; Vector Laboratories, Inc.) overnight at 4°C. The primary antibody was anti-Ki67 (dilution 1:500; ab15580; Abcam, USA). After washing with PBS, the tissues were incubated with Alexa Fluor 546 donkey anti-rabbit immunoglobulin G (dilution 1:500; Invitorogen Thermo Fisher Scientific, CA, USA) for 3 h at room temperature in a diluent (5% donkey serum containing 8% protein concentrate). Nuclear staining was performed with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) solution of fluorescent dye (Wako

Pure Chemical Industries).

Cell death assays

The sections were processed for terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay. The In Situ Cell Death Detection Kit TMR Red (Roche Applied Science, IN, USA) was used to perform TUNEL. The sections were treated with 4% paraformaldehyde fixative for 20 min and washed with PBS for 30 min. The sections were incubated for 2 min at 4°C with 20 μm/mL of the Proteinase K Solution (Invitrogen). Thereafter, the Enzyme Solution and Label Solution were mixed at a ratio of 1: 9, and the sections were reacted with the mixed solution for 60 min at 37°C. Finally, nuclear staining was performed with the DAPI solution of fluorescent dye.

Image analysis and cell counting

Tissue sections with HE staining and BrdU labelling were photographed using the BX 51TF optical microscope (Olympus) and those with TUNEL staining and Ki67 antigen (Ki67) labelling were photographed using the SP8 microscope (Leica, Wetzlar, Germany). Next, the images, except for HE staining ones, were imported into Photoshop (Adobe Photoshop CS6 extended, Adobe Inc., CA, USA), and the number of positively stained cells in the region of interests was counted according to the criteria

indicated in Supplementary Figure 1.

Statistical analysis

The differences among the means were evaluated by Student's two-tailed unpaired t-test. $P < 0.05$ and < 0.01 indicated statistical significance.

Results

The stage-dependent effects of CPA on the mandibular molar formation

To examine the effects of CPA administration at different stages of mandibular molars development, the mice received either CPA (100 mg/kg) or vehicle (control) at postnatal 1, 2, 4, 6, 8 and 10 days of age (Day 1, Day 2, Day 4, Day 6, Day 8 and Day 10, respectively), after which the mandibular molars at P26 were evaluated with micro-CT. The morphological abnormalities of the mandibular molars among the experimental groups receiving CPA in different timings were examined. Microdontia of M2 and absence of eruption of M3 were caused by the administration of CPA at Day 1 and Day 2 (Figures 1D–F), and the microdontia of M3 were observed by CPA administration at Day 8 (Figures 1P–R). Interestingly, the phenotype of dental anomalies was much severe in M2 than in M1 and M3 of the CPA-administrated group at Day 1 and Day 2 (Figures 1D–I). The agenesis of M3 was observed by CPA administration at Day 4 and

Day 6 (Figures 1J–O), but not at that of M1 and M2. The prevalence of agenesis by CPA administration is another novel finding of our experimental model involving CPA administration. Conversely, CPA administration to Day 10 mice showed neither missing teeth or microdontia nor absence of eruption (Figures 1S–U). In the experimental group, short roots were observed in all the administration timing of CPA, but not in the control group (Figure 1D–U). To assess the developmental stage of each mandibular molar at histological level, different chronologically aged mice mandibles were used to make serial sections.

According to the histological assessment of HE-stained sections at each time of administration, the developmental stage of M2 corresponded to the early bell stage at Day 1 to Day 2, late bell stage at Day 4 to Day 8 and root-forming stage at Day 10. Conversely, M3 was in the bud stage at Day 1–Day 2, cap stage at Day 4, early bell stage at Day 6–Day 8 and late bell stage at Day 10 (Supplementary Figure 2, Supplementary Table 1).

These results demonstrate that CPA administration induces different types and severity of dental anomalies that clearly correlate with the developmental stage of each tooth at the time of CPA treatment (Table 1). Notably, CPA administration to the mandibular molars at the cap and early bell stages led to the most severe dental anomaly phenotype

among the tooth developmental stages.

The dose-dependent effects of CPA on the mandibular molar formation

Next, we examined the dose-dependent effects of CPA on the morphological changes of M3 and the frequency of agenesis of M3 using 50 and 25 mg/kg of CPA at Day 4 and Day 6, wherein 100 mg/kg CPA-induced agenesis. The administration of 50 mg/kg CPA to mice at Day 4 resulted in microdontia in 87.5% of mice, whereas the administration of 50 mg/kg CPA to mice at Day 6 led to the loss of M3 in 50% (Table 2; Figures 2C, D, I, J). The administration of 25 mg/kg CPA to mice at Day 4 and Day 6 resulted in microdontia of M3, and the size of M3 was reduced at Day 6 rather than at Day 4 (Figures 2E, F, K, L). The size of M3 after CPA administration to mice at Day 4 was smaller at a dose of 50 mg/kg than at 25 mg/kg. These results again indicated time-dependent influence of CPA on tooth development because even at lower CPA doses, M3 that received CPA at Day 6 exhibited severe phenotype compared with Day 4. Moreover, linear phenotypic correlation indicated possible dose threshold of CPA for tooth agenesis at different developmental stages.

Histological changes of M3 after CPA administration

We examined the effects of CPA on the development of the mandibular molars. We found that the timing of CPA administration at Day 6 was the most susceptible one for the agenesis of M3 between Day 1 and Day 10. Therefore, we focused on the cellular mechanisms of how CPA leads to the agenesis of M3. The sections were obtained from 0.5 to 6 days (Day 6.5, Day 7, Day 8, Day 10 and Day 12) of 100 mg/kg CPA administration) to mice, starting at Day 6. In the mice at Day 6, M3 corresponded to the early bell stage during the formation of the distal cusp, and the crown calcification did not occur (Figures 3A and B). In the mice at Day 6.5, where the mice were at 0.5 days of CPA administration, no clear difference could be observed in M3 between the experimental and control groups (Figures 3C, D, M and N). In the Day 7 mouse at 1 day of CPA administration, the size of the tooth germ corresponding to the mandibular M3 was smaller in the experimental group than in the control group (Figure 3E, F, O and P). In the mice at Day 8, the distal cusp of M3 was not formed in the experimental group, and the arrangement of the cells of the inner and outer enamel epithelium was disturbed (Figures 3G, H, Q and R). Additionally, the cells in the dental papilla were sparse in the experimental group. In the control group at P10, the terminal differentiation of the inner enamel epithelium and dental pulp mesenchymal cells, followed by the matrix mineralisation, was observed. Conversely, the dental papilla of M3 in the experimental

group almost disappeared, and the regression of the internal and external enamel epithelium was observed (Figures 3I, J, S and T). In the control group at Day 12, mineralisation of the crown of M3 was evident and the tooth root formation of M3 had started, while M3 of the experimental group had almost disappeared (Figures 3K, L, U and V). From these results, the tooth germ corresponding to mandibular M3 of the experimental group began to shrink at 1 day of CPA administration at Day 7, followed by almost complete disappearance at 6 days of CPA administration at Day 12.

The effects of CPA administration on cell proliferation during tooth development of molars

To evaluate the effects of CPA administration on cell proliferation during molar development, immunostaining of Ki67 was performed. The number of Ki67-positive cells of M3 was counted at Day 6.5, Day 7, Day 8 and Day 10 from 0.5 to 4 days after 100 mg/kg CPA administration to mice at Day 6 (each group n = 3). In the control group, Ki67-positive cells were present in the inner enamel epithelium and the whole dental papilla of M3 from Day 6.5 to Day 8, and the number of Ki67-positive cells increased in the epithelial area and the dental papilla of M3 (Figures 4A–C and I–K). At Day 10 in the control group, Ki67-positive cells of M3 were present in the cervical loop

and its surrounding dental papilla, and the number of Ki67-positive cells decreased in both the epithelial area and the dental papilla of M3 (Figure 4D and I–K). Conversely, the number of Ki67-positive cells significantly decreased in the epithelial area and the dental papilla of M3 in the experimental group (Figures 4E–K). In the experimental group, the number of Ki67-positive cells per surface unit also decreased in the dental papilla (Figures 4L, Supplemental Table2). This phenomenon was confirmed by the analysis of BrdU incorporation (Supplementary Figure 3A–L).

We also evaluated the difference in proliferating cells during the development of M1 and M2 between the control and CPA groups. Compared with the control group, elevation of Ki67-positive cells around the cervical loop mesenchyme was observed in both M1 and M2 in the experimental group (Supplementary Figure 4A–H).

The effects of CPA administration on apoptosis during tooth development of molars

CPA induces cell death besides impairing the proliferation by arresting the cell cycle (7,10,11). Therefore, we performed TUNEL staining to detect apoptosis in M3 after CPA administration of 100 mg/kg in mice at Day 6. The number of TUNEL-positive cells per area and surface unit was counted at Day 6.5, Day 7, Day 8 and Day 10 from 0.5 to 4 days of 100 mg/kg CPA administration to mice at Day 6 (each group n = 3). In

the control group, only a few of the TUNEL-positive cells in M3 from Day 6.5 to Day 10 were observed in the epithelial area and the dental papilla, in contrast with the TUNEL-staining positive cells of M3 in mice from Day 6.5 to Day 7, which were observed around the outer enamel epithelium (Figures 5A–H). In the experimental group, the TUNEL-positive cells were observed in the epithelial area and the dental papilla of M3 at Day 6.5, and the number of TUNEL-positive cells per area and per surface unit significantly increased until Day 8 (Figures 5I–S). In the experimental group, the dental papilla of M3 at Day 10 almost disappeared, and the number of TUNEL-positive cells per area and per surface unit decreased to the baseline level (Figures 5L, P and Q–S). Supplementary Table 1 shows the detailed mean, SD and P value. These results showed that apoptosis in the tooth germ of M3 immediately increased after CPA administration, followed by a decrease at Day 10.

To evaluate the apoptosis of M1 and M2 after 100 mg/kg CPA administration in mice at Day 6, TUNEL staining was performed at Day 8. Compared with the control group, elevation in the number of TUNEL-positive cells around the cervical loop mesenchyme was observed in the experimental group for both M1 and M2 (Supplementary Figure 4I–P).

Discussion

Herein, we demonstrated the effects of CPA on the 3D morphological changes at different tooth developmental stages and doses during the tooth development of mandibular molars. Moreover, we found the serial histological changes in the tooth germ of the third molar after CPA administration to mice at Day 6. The present results also revealed the cell kinetics of the tooth germ during the process of agenesis or hypoplasia of M3 after CPA administration at Day 6.

3D morphological analysis by micro-CT demonstrated that CPA administration at different tooth developmental stages leads to various types of dental anomaly, including agenesis, microdontia and absence of eruption at each stage of tooth development (Figures 1 and 2). There was a clear correlation between the severity of the dental anomaly phenotypes and the tooth developmental stage at which the tooth germ was exposed to CPA (Figure 6). This finding is consistent with that reported previously, which indicated that high concentration of CPA administration is a risk factor for the incidence of dental anomalies of the teeth, particularly at a younger age (<5 years) (5). The patients with paediatric stem cell transplantation (SCT), who receive high-dose chemotherapy and total body irradiation as the preparative regimens for SCT, often have tooth agenesis (30). Reportedly, 52 SCT recipients (mean age at SCT 4.3 years) were

analysed for agenesis of permanent teeth, which occurred in 16 (31%) of 52 patients, and the most frequently missing teeth were second premolars (58%; 45 of 78 missing teeth), followed by second molars (28%), first premolars (10%) and upper lateral incisors (4%) (30). The second premolars, which are sensitive to SCT treatment with high-dose chemotherapy, reach the early bell to late bell stage at the age of 4–5 years; this is consistent with the time of SCT (30-32). Interestingly, the results showed that the second molar is the next most affected due to anticancer therapy; this developmental chronology resembles that of the second premolar (31,32). We also revealed that higher dosage of CPA administration resulted in more severe tooth phenotype (Figure 2). Collectively, the tooth developmental stage and CPA dosage are critical factors that determine the effects of CPA on tooth development.

Different susceptibilities for CPA in different tooth types are based on diverse developmental stage of teeth of same chronological age. For example, in mice, M1 begins to develop first among the three molars and continuously maintains the most progressed developmental stage (Supplementary Table 1). Similarly, in humans, there are substantial differences in developmental stages of individual teeth, which could also explain the phenotypic difference among different dentition. Notably, this is the first report to show agenesis of the molar after CPA administration in a rodent animal model.

CPA administration was previously conducted at older than Day 10, which corresponded to the root-forming stage in M2 and the late bell stage in M3 (23-26). This difference may explain why agenesis of the molar was not observed in previous studies. Thus, the present model enables to assess tooth developmental stage-specific effect of various environmental factors, such as that of CPA.

Histological assessment of tooth development in mice receiving CPA at Day 6 demonstrated shrinkage of the tooth germ in M3, which exposes CPA at the early bell stage, while there is no apparent overall morphological phenotype in M2, which exposes CPA at the late bell stage (Figure 3). Further analysis revealed that CPA exposure to the tooth germ at the early bell stage significantly inhibits cell proliferation in the inner enamel epithelium and significantly increased the apoptosis particularly in the stellate reticulum and dental papilla.

Dental papilla reportedly contains stem/progenitor cells of mesenchymal origin, and these cells demonstrate high proliferative activity (33). Our observations (Figure 7) collaborate with those of a previous report in that proliferative cells were observed in the dental papilla and enamel organ, except for enamel knots at the cap stage and in the inner enamel epithelium and dental papilla at the early bell stage, respectively (18). At the late bell stage, the proliferative cells were observed in both the cervical loops and

the inner enamel epithelium of the intercuspal areas. At later stages, the proliferative cells were integrated in Hertwig's epithelial sheath and the surrounding dental papilla. Cumulatively, high proliferative activity is required for morphogenesis, including rapid increase in the volume of the tooth germ, cusp formation and root extension (34-39).

Because proliferating cell populations are the prime targets of CPA, severe side-effects of CPA are associated with the occurrence of cell cycle arrest and apoptosis in several sensitive tissues at a rapid proliferation rate (40,41). Herein, TUNEL-positive cells were slightly increased in the inner enamel epithelium and dental papilla immediately after CPA administration, followed by peaking at 2 days post-administration. Conversely, the proliferative cells were decreased in the inner enamel epithelium and dental papilla at 24 h after CPA administration. These results align with those of a previous report demonstrating the cytotoxic effects of CPA on the taste buds (42). The disruption of proliferative cells in the tooth germ, particularly at the early bell stage, may lead to the shrinkage of tooth germ and finally result in the agenesis of the teeth. These data indicate that developing tooth germ contains proliferative cell population, which is highly susceptive to CPA, and that the proliferative cell population is essential for the normal development of a tooth.

On the other hand, cell proliferation and apoptosis rates for both M1 and M2 were

increased (Supplementary Figure 4I–P). Considering the results of CT analysis (Figure 1M-O), this increase in the rates around the cervical loop mesenchyme could be one of the reasons for root abnormalities in M1 and M2. Additionally, Figure 5 shows increased number of apoptotic cells in bone, potentially disrupting root growth and dentition eruption.

This is the first study that clearly demonstrates stage-dependent and dose-dependent effects of CPA administration during molar development. The stage-specific effect of CPA could rely on different cell proliferative profiles during each stage of tooth development. For example, M3 at Day 6 showed ubiquitous cell proliferation in the entire tooth germ, making this stage most susceptible for tooth agenesis of M3 by CPA administration (Figure 7). We demonstrated the cellular mechanisms of CPA-induced agenesis in M3. Our results indicate that CPA administration has a great impact on the cell kinetics of epithelial and mesenchymal cells, which may be the cause of dental anomalies after CPA administration in paediatric cancer treatment. Our data is expected to be useful for the better understanding of mechanistic basis of dental anomalies as the long-term side-effects of CPA treatment. Since adequate management and comprehensive care of the dentition by dental specialists is required to retain or improve the quality of life among paediatric cancer survivors, it may be necessary to inform

patients of the possible side-effects of CPA administration on tooth development and the future needs of dental treatment.

Conflict of interest

The author declare no competing interests.

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Figure legends

Figure 1. CPA administration induces various dental anomalies of the mandibular molars in a stage-dependent manner

Three-dimensional reconstruction images and slice images of the mandibular molars at P26 in the control group (A–C) and the experiment group (D–U). The mice received either CPA (100 mg/kg) or vehicle (control) at postnatal 1, 2, 4, 6, 8 and 10 days of age (Day 1, Day 2, Day 4, Day 6, Day 8 and Day 10, respectively). CPA administration at Day 1 and Day 2 caused microdontia of M2 and absence of eruption of M3 (D–I). Agenesis of M3 was observed by CPA administration at Day 4 and Day 6 (J–O). Microdontia of M3 is induced by CPA administration at Day 8 (P–R). Short roots observed in all CPA treated mice, but not in the control group (D–U).

CPA, cyclophosphamide; Day, postnatal Day; Day 1, Day 2, Day 4, Day 6, Day 8 or Day 10-CPA, Day 26 mice administered with CPA (100 mg/kg) at Day 1, Day 2, Day 4, Day 6, Day 8 or Day 10; arrowheads, microdontia; open arrowheads, absence of eruption; arrows, missing teeth; Bars 500 μm

Figure 2. CPA administration showing dose-dependent effects on the morphological changes of the mandibular third molar

Three-dimensional reconstruction images and the slice images of the mandibular molars at Day 26 in the experimental group. CPA administration of 100, 50 or 25 mg/kg at Day 4 (A–F) or Day 6 (G–L). The administration of 50 mg/kg CPA to mice at P4 frequently induces microdontia of M3, whereas the administration of 50 mg/kg CPA to mice at Day 6 often leads to the loss of M3 (C, D, I, J). The administration of 25 mg/kg CPA to mice at Day 4 and Day 6 cause microdontia of M3. Note that the size of M3 is smaller in Day 6 CPA mice than in Day 4 CPA mice (E, F, K, L).

CPA, cyclophosphamide; 100-CPA, CPA (100 mg/kg); 50-CPA, CPA (50 mg/kg); 25-CPA, CPA (25 mg/kg); Day, postnatal day; arrowheads, microdontia; arrows, missing teeth; Bars 500 μm

Figure 3. Development of the mandibular third molar

The left panels show haematoxylin and eosin-stained sections of the second molar (M2) and third molar (M3) (A, C, E, G, I, K, M, O, Q, S, U). The right panels show an enlarged view of M3 (B, D, F, H, J, L, N, P, R, T, V). The sections were obtained from 0.5 to 6 Days (Day 6.5, Day 7, Day 8, Day 10, Day 12) after 100 mg/kg CPA administration to mice, starting at Day 6. CPA, cyclophosphamide; 100-CPA, CPA (100 mg/kg); Day, postnatal day; Bars (A-V) 100 μm

Figure 4. Ki67 labelling in the sections of the third molar

CPA (100 mg/kg) was administrated to mice at Day 6, and the proliferative cells were labelled by anti-Ki67 antibody. The number of Ki67-positive cells in the third molar (M3) was counted at Day 6.5, Day 7, Day 8 and Day 10 (each group n = 3). In the control group, Ki67-positive cells were present in the inner enamel epithelium and the whole dental papilla of M3 from Day 6.5 to Day 8, and the number of Ki67-positive cells per area increased in the whole area of M3 (A–C, I–K). At Day 10 in the control group, Ki67-positive cells in M3 were present in the cervical loop and its surrounding dental papilla, and the number of Ki67-positive cells per area and surface unit decreased in both the whole area of M3 (D, I–L). The number of Ki67-positive cells per area and surface unit significantly decreased in the epithelial area and the dental papilla of M3 in the experimental group (E–L). Data are expressed as the mean ± standard deviation. CPA, cyclophosphamide; 100-CPA, CPA (100 mg/kg); Day, postnatal day; Bars 100 μm * $P < 0.05$, **$P < 0.01$

Figure 5. TUNEL staining in the sections of the third molar

After the CPA administration (100 mg/kg) to mice at Day 6, the apoptotic cells were

stained by TUNEL staining. The number of TUNEL-positive cells per area and surface unit in M3 in the control group (A–H) and the experimental group (I–P) was counted at Day 6.5, Day 7, Day 8 and Day 10 (each group n = 3). In the experimental group, TUNEL-positive cells were observed in the epithelial area and the dental papilla of M3 at Day 6.5, and the number of TUNEL-positive cells per area and surface unit were significantly increased until Day 8 (Fig. 7 I–S). In the experimental group, the dental papilla of M3 at Day 10 almost disappeared, and the number of TUNEL-positive cells decreased to the baseline level (Fig. 7 L, P, Q-S). Data are expressed as the mean ± the standard deviation. CPA, cyclophosphamide; 100-CPA, CPA (100 mg/kg); Day, postnatal day; Bars 100 μm *P < 0.05, **P < 0.01

Figure 6. The stage-dependent effects of CPA on the development of molars

Schematic illustrations indicating the relationship between the developmental stage of molars and dental anomalies induced by 100 mg/kg CPA administration.

CPA, cyclophosphamide; 100-CPA, CPA (100 mg/kg); red, proliferative cells

Figure 7. Ki67 labelling of the second and third molars during tooth development

Proliferative cells of the control group were labelled by anti-Ki67 antibody. In Day 1

mice, Ki67-positive cells were present in the tail tip and surrounding mesenchyme in M3 (A), and in M2, they were present in the inner enamel epithelium and whole dental papilla (B). Ki67-positive cells at Day 6 and Day 8 in mice were present in the inner enamel epithelium and whole dental papilla in M3 (C, E) and at Day 10 were present in the cervical loop and its surrounding dental papilla in M3 (G). Ki67-positive cells at Day 6, Day 8 and Day 10 in mice were present in the surrounding dental papilla of the Hertwig's epithelial root sheath cells in M2 (D, F, H). Bars (A–H) 100 μm.

Table 1. Stage-dependent effects of CPA on the morphological changes of the mandibular molar

CPA, cyclophosphamide; Day, postnatal Day; Timing, CPA (100 mg/kg) administration timing; M1, mandibular first molar; M2, mandibular second molar; M3, mandibular third molar

Table 2. Dose-dependent effects of CPA on the prevalence of agenesis of the mandibular third molar

The prevalence of agenesis of the mandibular third molar at Day 26 was calculated in the experimental group administered CPA (100 mg/kg, 50 mg/kg and 25 mg/kg) at P4

or P6 and the control group administered PBS at Day 4 or Day 6. CPA, cyclophosphamide; Day, postnatal day; Dose, CPA dose

Supplementary Figure 1. Diagram of the area counted for cell counting

A mesiodistal cervical loop of the third molar (M3) was connected by a straight line, and the inside of the line was defined as a dental papilla (A). A straight line parallel to the line of the cervical loop was drawn from the inflection point of the crown on the upper mesial side as the upper limit of the epithelial area (A). The number of stained cells was counted by dividing the epithelial area and the dental papilla of M3, and their sum was considered as the total number of tooth germ (A). The number of stained cells per surface unit was counted by defining 100 μm square unit of dental papilla in contact with the mesial cervical loop as shown in the figure B.

Supplementary Figure 2. Development of the second and third molars

Development of the mandibular third molar (M3) and second molar (M2) from Day 1 to Day 10. Haematoxylin and eosin staining sections of M2 and M3. In Day 1 and Day 2 mice, M3 is in the bud stage (A, C), and M2 is in the late bell stage with slight crown calcification (B, D). M3 in P4 mice is in the cap stage (E) and M3 in Day 6 and Day 8

mice is in the early bell stage (G). In Day 10 mice, M3 is in the late bell stage (I, K). In Day 4, Day 6 and Day 8 mice, M2 is in the late bell stage with crown calcification (F, H and J) and M2 in Day 10 mice is in the root formation stage (L). Bars (A–L) 100 μm.

Supplementary Figure 3. BrdU labelling in the sections of the third molar.

After CPA (100 mg/kg) administration to mice at Day 6, proliferative cells in the S phase were labelled by incorporating BrdU. The number of BrdU-positive cells per area and surface unit of the third molar (M3) was counted at Day 6.5, Day 7, Day 8 and Day 10 (each group n = 3). In the control group, BrdU-positive cells were present in the inner enamel epithelium and whole dental papilla of M3 from Day 6.5 to Day 8 and the number of BrdU-positive cells increased in the epithelial area and the dental papilla of M3 (A–C, I–K). The BrdU-positive cells of M3 at Day 10 were present in the cervical loop and its surrounding dental papilla, and the number of BrdU-positive cells per area decreased in the whole area of M3 (D, I–K). From Day 7 to Day 10 mice in the experimental group, the number of BrdU-positive cells per area and surface unit decreased in the whole area of M3 (F–L). The number of BrdU-positive cells in the tooth germ of M3 in the experimental group at Day 8 and Day 10 was significantly smaller than that in the control group (I–K). Data are expressed as the mean ± standard

deviation. CPA, cyclophosphamide; 100-CPA, CPA (100 mg/kg); Day, postnatal day; Bars 100 μm * P < 0.05, * * P < 0.01

Supplementary Figure 4. Ki67 labelling and TUNEL staining in the sections of the first and second molars.

CPA (100 mg/kg) was administrated to mice at Day 6, and the proliferative cells were labelled by anti-Ki67 antibody. The Ki67 labelling sections were observed at Day 8 (A–H). After the CPA administration (100 mg/kg) to mice at Day 6, the apoptotic cells were stained by TUNEL staining. The TUNEL staining sections were observed at Day 8 (I–P).The left panels show Ki67 labelling or TUNEL staining sections of the first molar (M1) and second molar (M2) (A, C, E, G, I, K, M, O). The right panels show an enlarged view of M1 and M2 (B, D, F, H, J, L, N, P). CPA, cyclophosphamide; 100-CPA, CPA (100 mg/kg); Day, postnatal day; Bars 100 μm

Supplementary Table 1. Developmental stages of the mouse mandibular molars

Supplementary Table 2. Statistical value of BrdU-, Ki67- and TUNEL-positive cell counting