In situ proliferation and differentiation of macrophages in dental pulp

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Received: 14 February 2011 / Accepted: 24 July 2011 / Published online: 16 September 2011
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Abstract The presence of macrophages in dental pulp is well known. However, whether these macrophages proliferate and differentiate in the dental pulp in situ, or whether they constantly migrate from the blood stream into the dental pulp remains unknown. We have examined and compared the development of dental pulp macrophages in an organ culture system with in vivo tooth organs to clarify the developmental mechanism of these macrophages. The first mandibular molar tooth organs from ICR mice aged between 16 days of gestation (E16) to 5 days postnatally were used for in vivo experiments. Those from E16 were cultured for up to 14 days with or without 10% fetal bovine serum. Dental pulp tissues were analyzed with immunohistochemistry to detect the macrophages and with reverse transcription and the polymerase chain reaction (RT-PCR) for the detection of factors related to macrophage development. The growth curves for the in vivo and in vitro cultured cells revealed similar numbers of F4/80-positive macrophages in dental pulp tissue. RT-PCR analysis indicated the constant expression of myeloid colony-stimulating factor (M-CSF) in both in-vivo- and in-vitro-cultured dental pulp tissues. Anti-M-CSF antibodies significantly inhibited the increase in the number of macrophages in the dental pulp. These results suggest that (1) most of the dental pulp macrophages proliferate and differentiate in the dental pulp without a supply of precursor cells from the blood stream, (2) M-CSF might be a candidate molecule for dental pulp macrophage development, and (3) serum factors might not directly affect the development of macrophages.

Keywords Dental pulp · Immunohistochemistry · Macrophage · M-CSF · Organ culture · Mouse (ICR)

Introduction

Macrophages are a heterogenous population of cells, characterized by their morphology, function, and metabolism. At least two types of macrophages are distributed in the body: resident and exudative macrophages (Daems and Brederoo 1972). Whereas exudative macrophages migrate into sites of inflammation in response to several chemokines, resident macrophages are ubiquitously distributed around the body under normal healthy conditions (Daems and Brederoo 1973; Daems et al. 1976, 1979; Soranzo et al. 1978; Daems and van der Rhee 1980). According to the concept of the mononuclear phagocyte system (van Furth et
al. 1972), both these types of macrophages are derived from blood monocytes, which originate in the bone marrow. Furthermore, macrophages are generally accepted to be relatively short-lived cells and do not show any proliferative activity (van Furth 1975, 1980, 1989, 1992). However, several recent studies have demonstrated the in situ proliferation of resident macrophages in various tissues in response to several cytokines, such as myeloid colony-stimulating factors (GM-CSF and M-CSF), secreted into the proliferation microenvironment (Naito et al. 1996; Sawa et al. 2003; Douglass et al. 2008).

Large numbers of macrophages exist in the dental pulp and are considered to be the major immunocompetent cells that fight against bacterial infections caused by dental caries (Nakakura-Ohshima et al. 2003; Zhang et al. 2006). In reality, macrophages migrate toward the infection site in pulpitis. However, whether dental pulp macrophages proliferate and differentiate in situ, or whether monocytes derived from the circulation differentiate into macrophages remains uncertain.

The tissue organ culture system is commonly used to examine the development of the tooth organ (Fujiwara 1997; Fujiwara et al. 2005). Our previous studies have indicated that 2 days of development in culture is almost equal to 1 day of in vivo development, and that the calcification of dentin and enamel can be detected following culture under either serum-supplemented or serumless conditions (Evans et al. 1988; Nakamura et al. 1994). In the system presented here, the migration of blood monocytes from the circulation can be excluded, because of the isolation of tooth organs from the mandibles.

In this study, we have used immunohistochemistry to examine the number and phenotype of macrophages in the dental pulp as a product of time in order to determine whether these cells proliferate and differentiate in situ. We have also used reverse transcription with the polymerase chain reaction (RT-PCR) to analyze the possible factors responsible for the proliferation and differentiation of these macrophages.

Materials and methods

The experimental protocol used in this study was reviewed and approved by the Animal Care Committee of Showa University.

Mice

Pregnant ICR mice were purchased from Sankyo Laboratory Service (Tokyo, Japan) and maintained under routine conditions at the Laboratory Animal Center of Showa University. Mandibular first molars taken from mice aged from 16 days of gestation (E16) to 5 days postnatally (5dPN) were examined.

Organ culture

E16 mandibular first molar tooth organs were dissected and maintained for up to 14 days in a Tronwell culture system with serum-supplemented or a serumless, chemically defined medium as previously described (Evans et al. 1988; Nakamura et al. 1994). The explants were placed on 0.2-μm pore-size Millipore filter discs (Bedford, Mass., USA) and supported by stainless steel mesh triangles. The explants were cultured in Grobstein Falcon dishes under optimal humidity conditions in an atmosphere containing 5% CO2 and 95% air. The medium used was Fitton-Jackson modified BGJb medium (Sigma, St. Louis, Mo., USA), with or without 10% FBS (fetal bovine serum), and supplemented with 100 mM ascorbic acid, 100 U/ml penicillin, and 100 mg/ml streptomycin. The medium (pH 7.4 at the start of each culture) was changed every other day.

To examine the inhibitory effect of M-CSF on the development of dental pulp resident macrophages, a neutralizing antibody against M-CSF (R & D Systems, Abingdon, Berkshire, UK) was added to the culture medium at a final concentration of 16 μg/ml. For control experiments, isotype-matched rat IgG2b (eBioscience, Burlingame, Calif., USA) was added to the medium at the same concentration.

Tissue preparation

Mandibles from E16 to 5dPN mice and first molar tooth organs cultured for 2–14 days were fixed with 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS) at pH 7.4. After decalcification with 10% EDTA, specimens were immersed in 5%, 15%, and 30% sucrose, embedded in Tissue–Tek O.C.T Compound (Sakura Finetek USA, Torrance, USA), and snap-frozen in a mixture of acetone and dry ice for analysis by immunohistochemistry. Sections (10 μm thick) were processed for hematoxylin and eosin staining and immunohistochemistry.

Antibodies

Four markers were applied in this study for the identification of macrophage-lineage cells in the dental pulp: F4/80, CD68, ER-MP20, and ER-MP58. F4/80 is usually used as the marker for resident macrophages, whereas CD68 is a marker of macrophage activation (Leenen et al. 1994; Dambach et al. 2002; Lloyd et al. 2008). ER-MP20 and ER-MP58 are both used as markers for monocytic progenitor cells (Leene et al. 1990, 1994; Chan et al. 1992).
1998; Kennedy and Abkowitz 1998). Proliferating cell nuclear antigen (PCNA) was used as a proliferation marker (Hillmeister et al. 2008; Vassiliou et al. 2010).

Anti-F4/80, CD68, ER-MP20 (Ly-6C), ER-MP58, and PCNA monoclonal antibodies were purchased from SEROTEC (Oxfordshire, UK). Goat anti-rat IgG antibodies, conjugated with either Alexa Fluor 488 or Alexa Fluor 594, were purchased from Molecular Probes (Eugene, Ore., USA). Biotinylated goat anti-rat IgG antibody was purchased from VECTORS (Burlingame, Calif., USA).

Immunohistochemical procedures

Frozen sections (10 μm thick) were cut from the embedded tissues, placed on poly-L-lysine-coated glass slides, and air-dried. After incubation in 0.3% H2O2-methanol for 30 min, the sections were incubated with 5% normal goat serum in PBS containing 5% bovine serum albumin and 0.025% Triton X-100, followed by incubation with each of the monoclonal antibodies. After several rinses, the sections were incubated with biotinylated goat anti-rat IgG antibody and then with an avidin-biotin-bound horseradish peroxidase complex. After being washed, the sections were incubated with a mixture of 3,3′-diaminobenzidine tetrahydrochloride (0.5 mg/ml; WAKO, Osaka, Japan) and H2O2 at a final concentration of 0.03% in 0.1 M TRIS-HCl buffer at pH 7.6. Counterstaining was achieved with methyl green. As controls, sections were incubated with either normal rat serum or PBS instead of the primary antibody. After the immunolabeling step, the number of F4/80-, CD68-, ER-MP20-, and ER-MP58-positive cells in the dental pulp was counted (100×100 μm).

For the double-labeling of F4/80- and CD68- or ER-MP20-positive macrophages, sections were first incubated with the F4/80 antibody and then with the Alexa-Fluor-488-conjugated goat anti-rat IgG antibody. After being washed, sections were incubated with anti-CD68 or anti-ER-MP20 antibody and then with Alexa-Fluor-594-conjugated goat anti-rat IgG antibody. For the double-labeling of PCNA- and F4/80-positive macrophages, sections were first boiled with antigen retrieval buffer (10 mM sodium citrate, pH 6.0) for 10 min before the sections were incubated with anti-mouse-PCNA mouse monoclonal antibody (BD Biosciences Pharmingen, San Diego, Calif., USA) and then with Alexa-Fluor-488-conjugated goat anti-mouse IgG antibody. After being washed, sections were incubated with anti-F4/80 antibody and then with Alexa-Fluor-594-conjugated goat anti-rat IgG antibody. After several washes with PBS, sections were mounted with AquaMount (Polysciences, Warrington, Pa., USA) and observed with a Nikon ECLIPSE 50i fluorescent microscope (Tokyo, Japan).

RT-PCR analysis

The in vivo and in vitro cultured mandibular first molar tooth organs were homogenized after the careful removal of enamel organs under a dissection microscopy. Total RNA was extracted from each sample by using an RNeasy Mini kit (QIAGEN, Tokyo, Japan). A total of 4 μg RNA served as a template for RT-PCR, which was performed by means of an Gene Amp Gold RNA PCR (Applied Biosystems, Tokyo, Japan) with mG-CSF, mGM-CSF, and mM-CSF sequence-aligned primers. The primers used for PCR analysis were as follows: GM-CSF forward, 5′-GCG TGA CAT TAA AGA AGC TG-3′; reverse, 5′-CTC AGG AGG AGC AAT GAT CTT G-3′; G-CSF forward, 5′-GGG ACA AGA CAT CCC TGT TT-3′; reverse, 5′-CTG TGA GGA CAG GAA ACC CT-3′; M-CSF forward, 5′-CTA GGG GCC AGC ATT AGA CC-3′; reverse, 5′-GAC ACA TAC ACC CCA GAC G-3′; and bet-actin forward, 5′-GCC AGC ATT AGA CC-3′;

Statistical analyses

Statistical significance was evaluated by using the Student’s unpaired t-test or Mann-Whitney U test, for which P values of <0.01 were considered significant.

Results

Immunohistochemical detection of resident macrophages in dental pulp during in vivo development

In the dental pulp of the first mandibular molar tooth organ at age E16, dental pulp cells adjacent to the basement membrane became polarized, whereas no dentin matrix was secreted at this time (Fig. 1a). A small number of F4/80-, ER-MP20- (Ly-6C), and ER-MP58-positive cells were detected, whereas CD68-positive cells were not detected at this stage (Fig. 1e, i, m, q). At 0dPN, dental pulp cells beneath the basement membrane differentiated into odontoblasts and secreted dentin matrix (Fig. 1b). F4/80- and ER-MP20-positive cells were observed throughout the dental
pulp (Fig. 1f, n), and CD68- and ER-MP58-positive cells were also detected at this stage (Fig. 1j, r). During this development, the odontoblasts secreted more dentin matrix and formed calcified dentin (Fig. 1c, d). Inner enamel epithelial cells differentiated into secretory ameloblasts and formed enamel (Fig. 1c, d).

The number of F4/80- and CD68-positive cells increased significantly with development (Fig. 1g, h, k, l; see also quantitation below). In contrast, the number of ER-MP20- and ER-MP58-positive cells decreased or were constant at a low level (Fig. 1o, p, s, t; see also quantitation below). At all of the developmental stages, the number of CD68-positive cells was lower than that of the F4/80-positive cells (see quantitation below).

These results suggest that the macrophages actively proliferate within the dental pulp, even if a relatively low number of monocytes might penetrate from the bloodstream.
Immunohistochemical detection of resident macrophages in in-vitro-cultured dental pulp

First mandibular molar tooth organs taken from mice aged E16 were used for organ culture. When cultured in serum-supplemented media, tooth organs developed with time. By 6 days, odontoblasts secreted dentin matrix (Fig. 2a), and the formation of dentin and enamel proceeded at 10 and 14 days (Fig. 2b, c). The number of F4/80-positive cells increased significantly during development (Figs. 2d–f, 3b). CD68-positive cells were detected from 6 days of culture, and the number of these cells increased significantly during development (Figs. 2g–i, 3c). ER-MP20- and ER-MP58-positive cells decreased significantly during development (Figs. 2j–o, 3d, e). The number of F4/80-positive cells was always higher than that of the CD68-positive cells (Fig. 3b, c).

The same results were obtained from the organ culture experiments in the serumless, chemically defined media condition (Fig. 3b, c). No significant differences were detected between the numbers of F4/80- and CD68-positive cells under either of the two culture conditions (Fig. 3b, c). The number of these cells in vivo was higher than the organ culture tooth organ cells (Fig. 3a–c).

The organ culture results strongly support the possibility of the direct proliferation of macrophages within the dental pulp and also indicate the lack of contribution from serum factors for macrophage development.

Double-immunostaining of macrophages

Double-staining of the macrophages with the anti-F4/80 and anti-CD68 antibodies in vivo and in vitro showed that

**Fig. 2** Development of E16 mouse mandibular first molars in organ culture under serum-supplemented conditions for 6 days (a, d, g, j, m), 10 days (b, e, h, k, n), and 14 days (c, f, i, l, o). H-E staining indicated the development of tooth organs (a–c). Immunohistochemical detection of F4/80-positive cells (d–f), CD68-positive cells (g–i), ER-MP20-positive cells (j–l), and ER-MP58-positive cells (m–o) indicated an increase in the number of macrophages with tooth organ development. Bar 100 μm
all CD68-positive cells were also F4/80-positive, whereas the F4/80 single-positive cells were distributed throughout the dental pulp (Fig. 4c, f). Most of the macrophages were relatively large and had well-developed cell processes (Fig. 4a-f).

Double-staining of the macrophages with anti-F4/80 and anti-ER-MP20 antibodies in vivo and in vitro showed that all F4/80-positive cells were also ER-MP20-positive at age E16 (Fig. 4g-i). During development, the number of F4/80 single-positive cells increased, whereas the number of ER-MP20 single-positive cells decreased. As a result, the number of double-positive cells decreased (Figs. 3a, d, 4j-r). These results suggest that immature ER-MP20-positive cells present in the interstitial area within the dental pulp differentiate into F4/80-positive cells via the ER-MP20 and F4/80 double-positive developmental stage.

Double-immunostaining of macrophages with anti-F4/80 and anti-PCNA antibodies

As the results from the in vivo and in vitro immunostaining strongly suggested the direct proliferation of F4/80-positive cells within the dental pulp, we next examined the expression of PCNA in F4/80-positive cells. Double-staining of macrophages with anti-F4/80 and anti-PCNA antibodies in vivo and in vitro revealed that some of the F4/80-positive cells were also PCNA-positive (Fig. 5), which indicated that the F4/80-positive cells were proliferating within the peripheral dental pulp.

RT-PCR analysis of dental pulp

Next, we examined the factors relating to the development of macrophages in the dental pulp. The expression of three cytokines important for leukocyte development (G-CSF, GM-CSF, and M-CSF) was examined by RT-PCR (Fig. 6). Among these three cytokines, the high expression of M-CSF was detected constitutively during the examined period in both in vivo and in vitro dental pulp cells. The expression of GM-CSF was detected at all of the examined times, although this expression was not stable. G-CSF was also expressed constantly during the developmental period.

Effects of M-CSF-neutralizing antibody on macrophage development in dental pulp

Finally, we examined the effect of M-CSF on macrophage development in dental pulp by means of the organ culture system. The addition of anti-M-CSF in the culture medium...
resulted in a significant reduction of F4/80-positive and CD68-positive cells in the dental pulp compared with the isotype matched control at day 12 of culture ($P < 0.01$; Fig. 7). No obvious developmental defects could be detected in the tooth organs following the addition of the antibody (data not shown).
Discussion

In this study, we used the tooth organ culture system to examine the proliferation of macrophages within the dental pulp. For cultivation, tooth organs were isolated from the mandibles, a procedure that resulted in the exclusion of a blood monocyte invasion from the circulation. In the tooth organ culture system, the number of macrophages in the dental pulp increased during organ development. At age E16, the dental pulp contained a few F4/80-positive macrophages. Even if these F4/80-positive cells had migrated into the dental pulp from the bloodstream as monocytes, our results provide direct evidence that the macrophages proliferate in the dental pulp in situ.

The number of macrophages was always higher in vivo than in vitro. As blood vessels were developed in the dental pulp of in vivo tooth organs, the monocytes must have migrated from the bloodstream into the dental pulp. This environmental difference might explain the difference between the number of macrophages observed under the two conditions. However, the number of ER-MP20-positive cells gradually decreased during development. In contrast, the number of F4/80-positive macrophages increased during development. Furthermore, under the in vivo and

Fig. 5 Double-immunostaining of dental pulp macrophages with anti-PCNA (a, d) and anti-F4/80 (b, e) antibodies in 4dPN (a–c) and 8-day-cultured tooth organs (d–f). All PCNA-positive cells were F4/80-positive (c, f). Bar 25 μm

Fig. 6 RT-PCR analysis of the expression of M-CSF, GM-CSF, G-CSF, and β-actin in dental pulp of in vivo and organ culture with or without fetal bovine serum (w/FBS, w/oFBS)
in vitro culture conditions, F4/80-positive macrophages were also PCNA-positive, and the number of F4/80-positive cells was always higher than the CD68-positive cells. These results strongly imply that, even if a small number of macrophages is supplied from the bloodstream, most of the F4/80-positive macrophages in the dental pulp represent a self-proliferating population that is distinct from the monocyte population and that develops into functionally activated CD68-positive cells in situ.

Several studies have suggested that macrophages proliferate in various organs. During ontogeny, primitive macrophages develop, proliferate, and differentiate into fetal macrophages before the production of monocytes in the fetal hematopoietic tissues (Naito and Wisse 1977; Naito et al. 1986, 1989, 1990, 1991; Takahashi et al. 1989; Higashi et al. 1992). Yamamoto et al. (2008) have identified macrophages in osteopetrotic (op/op) mice. These animals contain approximately half the number of macrophages found in healthy animals. Resident macrophages are capable of proliferating under various experimental conditions (Wisse 1974; Widmann and Fahimi 1975; Hibbs et al. 2007). In mice that have been rendered monocytopenic following the administration of strontium-89 (Yamada et al. 1990; Naito and Takahashi 1991), the number of Kupffer cells is maintained by the proliferation of resident Kupffer cells.

The production of monocytes and macrophages is controlled by various cytokines, such as interleukin-6 (IL-6), IL-3, GM-CSF, and M-CSF. Among these, M-CSF is the most important key molecule for mediating the development and differentiation of a restricted macrophage lineage belonging to the mononuclear phagocyte system and for the production of heterogeneous macrophage populations (Rutherford et al. 1993; Naito et al. 1996; Douglass et al. 2008). Moreover, M-CSF has been reported to stimulate the growth of macrophages, and resident macrophages exist in the local tissues, such as the Kupffer cells in the liver, microglial cells in the brain, mesangial cells in the kidney, and osteoclasts in bone (Takashima et al. 1995; Douglass et al. 2008). M-CSF-derived macrophages are larger in size, develop more abundant intracellular organelles, and extend more well-developed cell processes than GM-CSF-derived and IL-3-derived macrophages (Morioka et al. 1994).

The macrophages detected in the dental pulp in this study were also large and extended several cell processes. RT-PCR analysis also revealed the constitutive expression of M-CSF mRNA in the dental pulp, as previously indicated by Sawa et al. (2003). The lack of F4/80-positive resident macrophages in the dental pulp was furthermore indicated in osteopetrotic (op/op) mice, which carried an M-CSF mutation (Nagahama et al. 1998). Neutralizing antibodies against M-CSF significantly inhibited the proliferation of resident macrophages in the dental pulp in our study. Tooth organs developed in an organ culture system in both serum-supplemented and serum-less conditions. Under both conditions, the number of F4/80-positive cells increased similarly. These results indicate that M-CSF is the main cytokine responsible for the development of resident macrophages in the dental pulp.

Macrophages in the dental pulp are considered to be one of the major types of immunocompetent cells that fight dental pulp infections. Several studies have mentioned the possibility of resident macrophages being involved in the regulatory function and differentiation of odontoblasts (Ohshima et al. 1995; Tsuruga et al. 1999; Nakakura-Ohshima et al. 2003). However, the precise functions of the macrophages in the dental pulp have not yet been clarified. According to Zhang et al. (2006), two types of cells with well-developed cell processes exist in the dental pulp: the CD11c-positive sentinel and F4/80-positive interstitial cells. The CD11c-positive cells constitutively express toll-like receptors 2 and 4 and displayed high migration activities. They rapidly move to the infected regions in the dental pulp and then migrate into the local lymph nodes. In contrast, F4/80-positive cells exhibit low migration activity. Therefore, CD11c-positive cells and F4/80-positive cells are considered to be sentinel and interstitial cells, respectively. In our study, we have not examined the further phenotypic characterization of resident macrophages. Whether these cells show the same phenotypic and functional characterizations in vivo and in vitro remains undetermined and should be considered in future investigations.

In conclusion, our in vivo and in vitro studies indicate that dental pulp macrophages proliferate and differentiate in situ. The main factor involved in the development of these macrophages might be M-CSF.
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