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Pro-Inflammatory Cytokines Induce Odontogenic Differentiation of Dental Pulp-Derived Stem Cells

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ABSTRACT

Postnatal dental pulp stem cells (DPSCs) represent a unique precursor population in the dental pulp, which have multipotential and harbor great potential for tissue engineering purposes. However, for therapy applications, transplanted cells are often exposed to unfavorable conditions such as cytokines released from necrotic or inflammatory cells in injured tissues. It is not clear how stem cells exposed to these conditions changes in their characteristics. In this study, the effects of pro-inflammatory cytokines, such as IL-1 and TNF, on DPSCs were investigated. Cells were treated with IL-1, TNF, or both for 3, 7, and 12 days. The cultures were evaluated for cell proliferation, ALP activity, and real-time PCR. We found that a short treatment (3 days) of pro-inflammatory cytokines induced the odontogenic differentiation of DPSCs. Furthermore, post 3 days treatment with pro-inflammatory cytokines, the cell–scaffold complexes were implanted subcutaneously in mice for 8 weeks. Histological analysis demonstrated that the cultures gave obviously mineralized tissue formation, especially for both IL-1 and TNF applied. These data suggest that IL-1 and TNF produced in the early inflammatory reaction may induce the mineralization of DPSCs. J. Cell. Biochem. 113: 669–677, 2012.

KEY WORDS: DENTAL PULP STEM CELLS; CYTOKINE; DIFFERENTIATION; INFLAMMATION

Tooth loss, caused by trauma, periodontal disease, and hypoplasia, is considered a major health problem. Damaged teeth are now commonly restored or replaced by synthetic materials. Still, the development of natural tooth substitutes may be a prospect with the emergence of tissue engineering techniques, especially the recent isolation of postnatal dental pulp stem cells (DPSCs) [Gronthos et al., 2000; Miura et al., 2003; Shi and Gronthos, 2003].

Currently, studies have already demonstrated that DPSCs, as a kind of mesenchymal stem cells (MSC), exhibited multilineage potential and could give rise to dentin–pulp complex-like structures or a woven bone like structure in vivo [Gronthos et al., 2002; Zhang et al., 2005; Yang et al., 2009ab]. All these studies confirm that DPSCs might be a candidate cell type for dental tissue engineering and the potential feasibility for dental tissue repair or replacement by regenerating dental substitutes.

However, when stem cells are transplanted for cell replacement therapy, transplanted stem cells mostly are exposed to unfavorable conditions such as hypoxia or cytokines released from necrotic or inflammatory cells. Then, some changes in their characteristics could happen. In fact, it has been reported that the differentiation potential and cytokine expression of MSCs are altered under hypoxic conditions [Fink et al., 2004]. Some pro-inflammatory cytokines have been reported to be involved in alterations of stem cell function as well as recruitment of inflammatory cells or immune cells by injured tissues [Kim et al., 1998]. In particular, interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) play important roles in the inflammation response and might be markers of early inflammation [Kjeldsen et al., 1993; Cotran et al., 2002]. Pro-inflammatory cytokines, such as TNF-α and IL-1β, have been shown to affect osteoclastogenesis or bone formation [Lisignoli et al., 2004]. Although the mechanism is not clear, extrinsic stimuli from bacteria, like lipopolysaccharide (LPS) and peptidoglycans (PGN), can promote osteogenic differentiation of MSCs with an increasing TNF-α expression [Cho et al., 2006].

Pro-inflammatory cytokines, such as TNF and IL-1, have also been found to be highly expressed in inflamed pulp tissues [Stashenko et al., 1998; Coil et al., 2004]. In experimentally induced rat pulpitis, large amounts of IL-1 and TNF-α have been identified in dental pulp fibroblasts [Tani-Ishii et al., 1995]. Further study demonstrated that the early inflammatory reaction is a protective mechanism in pulp cells. IL-1 induces the expression of mineralization markers in dental pulp cells. However, long-term treatment of TNF-α may inhibit the cell mineralization [Min et al., 2006].

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DPSCs are specific MSCs that exist in human dental pulp tissues. DPSCs may have excellent potential for dentin repair and hard tissue regeneration. In response to the appropriate stimuli, DPSCs may differentiate into odontoblasts and form reparative dentin by defensive pulp reactions. Dentinogenesis is a cell-mediated process that is regulated by growth factors and cytokines and is modulated by alterations in the extracellular environment. Hence, before these cells can be used for clinical therapy, it is critical that we need to understand their biological properties in response to extrinsic and intrinsic stimuli. Especially, the effects of pro-inflammatory cytokines should be clarified. Local inflammation stage may bring different effects on odontoblast differentiation and dentin repair. Therefore, in this study, we aimed to investigate the effects of IL-1 and TNF-α on DPSCs. We hypothesized that IL-1 and TNF-α produced in the early inflammatory reaction may induce the osteogenic differentiation and mineralization formation of DPSCs in vitro and in vivo.

MATERIALS AND METHODS

CELL CULTURES

Rat DPSCs were selected on the stem cell marker STRO-1 by fluorescence-activated cell sorting (FACS) as described before [Yang et al., 2007a]. Briefly, 10×10^6 primary rat incisor dental pulp cells were resuspended in 1 ml PBS/1% BSA and preincubated for 15 min on ice. Subsequently, cells were incubated with 200 μl PBS/1% BSA with STRO-1 monoclonal antibody (20 μg/ml, R&D Systems, Minneapolis, MN) for 15 min on ice. After centrifugation and washing, samples were incubated in 200 μl PBS/1% BSA with phycoerythrin (PE)-conjugated goat anti-mouse IgG antibody (1:50; Sigma) for an additional 15 min on ice, washed, resolved, and kept on ice until sorting. Cells were sorted using a FACStar Plus flow cytometer (Beckton Dickinson & Co., Mountain View, CA). The samples were maintained at 4°C through the cell-sorting procedures. Positivity was defined as a level of fluorescence greater than 99% of the blank (without 1st and 2nd antibody reaction) and negative (without 1st antibody reaction) controls. STRO-1 selected cells were expanded to the 10th passage in minimal essential medium-α (α-MEM; GIBCO-BRL) supplemented with 10% fetal calf serum (FCS; GIBCO-BRL) and gentamycin (50 μg/ml; Sigma). Further, immunofluorescence microscopy was used to confirm the STRO-1 positive cell content after the expansion step. DPSCs were characterized as described previously [Yang et al., 2007ab].

CYTOKINE TREATMENT

The rat DPSCs were seeded in 24-well plate at a density of 2×10^4 cells in 1 ml of culture medium per well. Upon reaching 80% confluence, cells were used for cytokine treatment. Cells were cultured in the medium as described with or without IL-1β and TNF-α (Sigma). In this study, 3, 7, and 12 days cultures were performed as a short-term, middle-term, and long-term culture, respectively. The final concentration of IL-1β and TNF-α used on this study was 10 ng/ml [Min et al., 2006]. Culture medium was replaced at 24-h interval.

CELL PROLIFERATION AND CELL VIABILITY

At day 3, 7, and 12 with cytokine treatment the proliferation rates of the cells, as measured by the total DNA content ($n = 6$), were assessed with a PicoGreen dsDNA quantification kit (Molecular Probes, Eugene, OR) as described previously [Zhang et al., 2005].

Cell viability was assessed using a commercially available cytotoxicity assay, according to the instructions of the manufacturer (Promega Corporation, Madison, WI). The test is based on the conversion of a tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide, MTT) into a formazan product. Briefly, 48-h post-transfection ($n = 6$), 150 μl dye solution was added to each well, and cells were incubated at 37°C for 4 h. Subsequently, 1 ml of solubilization/stop solution was added to each well. The plate was incubated at room temperature for another hour then measured at 570 nm using an ELISA microplate reader (Bio-Tek Instruments Inc) [van den Beucken et al., 2006].

ALP ACTIVITY

The same supernatants as used for the PicoGreen assay were also used to measure cellular ALP activity (Sigma). Twenty microliters of 0.5 M 2-amino-2-methyl-1-propanol (AMP) buffer was added to 80 μl of the samples or standards. Next, 100 μl of substrate solution (*p*-nitrophenyl phosphate) was added and the mixtures were incubated at 37°C for 1 h. ALP activity was measured at 405 nm using an ELISA microplate reader (Bio-Tek Instruments Inc).

PROTEIN ASSAY

The amounts of osteocalcin (OC) and bone sialoprotein (BSP) were measured by ELISA kits (Cusabio Biotech, China) according to the instructions of the manufacturer. Briefly, after washing twice in PBS, each well added 1 ml of deionized water to lyse the cells. Then, the supernatants were collected and assayed according the manufacture instruction. A rat OC or BSP standard solution was diluted to generate a standard curve (0–50 ng/ml).

REAL-TIME QUANTITATIVE PCR

Total RNA of each sample was prepared with a Qiagen RNEasy kit (Qiagen, Valencia, CA). Then, 1 μg of total RNA was used to perform the reverse transcriptase (RT) reaction. The obtained cDNA was used as a template in PCR. The odontogenic differentiation of cells was monitored by analyzing the specific markers alkaline phosphatase (ALP), OC, BSP, dentin sialophosphoprotein (DSPP), and dentin matrix protein 1 (DMP-1). GAPDH was used as house-keeping gene to normalize RNA expression. The rat-specific primers used in the current study were as described previously [Yang et al., 2007c]. Real-time PCR was performed using the SYBR Green PCR kit (Qiagen), and controlled in a spectrofluorimetric thermal iCycler® (Bio-Rad, Hercules, CA). After the real-time PCR run, the C_t-value showed how many PCR cycles were necessary to obtain a certain level of fluorescence. Amplification efficiency of different genes was determined relative to GAPDH as an internal control ($\Delta C_t = C_t$ gene $- C_t$ GAPDH). The mRNA in each sample was calculated by the comparative $\Delta\Delta C_t$ (ΔC_t gene – ΔC_t control) value

method [Livak et al., 2001]. Each measurement was assessed in triplicate.

THREE-DIMENSIONAL SCAFFOLD CULTURE

For the in vivo analysis, sintered HA/TCP Camceram® ceramic disks of a 6 mm diameter and 3 mm thickness with a HA/TCP ratio of 60/40 were used (CAM Implants, Leiden, The Netherlands). The volumetric porosity of the disks was 90%. All scaffolds were prewetted in α-MEM overnight. Then, DPSCs were seeded onto the scaffolds by submerging the scaffolds in a suspension of 5.0×10^6 cells/ml while gently rotating at 6 rpm for 3 h. For every six scaffolds, 1 ml cell suspension was used. Subsequently, the scaffolds were put in 24-well plates with 1 ml of medium. The cell–scaffold complexes were cultured with or without IL-1β and TNF-α (10 ng/ml).

On day 3 post-treatment, cell/scaffold complexes were rinsed with filtered PBS, and fixed in 2% glutaraldehyde for 5 min, dehydrated in graded series of ethanol, and dried in tetramethylsilane (Merck, Germany). Gold was sputtered on the samples just before SEM analysis. The specimens were examined with a JEOL 6310 SEM (n = 2). For energy-dispersive spectroscopy (EDS) measurement, samples like those for SEM (but without sputtering gold) were analyzed.

IN VIVO ANALYSIS

On day 3 post-treatment, four samples in total of each group were used for in vivo implantation. Sixteen 10-week-old BALB/c immunocompromised nude mice were used after approval from the Wuhan University Animal Ethics Committee. National guidelines for the care and use of laboratory animals were obeyed. Surgery was performed under general anaesthesia (isoflurane 1.5–3%). The backs of the mice were washed and disinfected with povidone–iodine. On each flank, parallel to the spinal column, two small incisions of about 10 mm were made through the skin. Using blunt dissection, subcutaneous pockets were created. To avoid possible cross-over effects, each mouse received always four implants of the same group. After implant placement the wounds were closed using staples. Eight weeks post-implantation, the samples were retrieved.

The samples for each group were fixed in formalin, dehydrated in a series of ethanol, and embedded in methylmethacrylate (MMA). After polymerization of the MMA, at least three thin transversal sections (10 μm) of each tissue block were made using a modified sawing microtome technique. The sections were stained with methylene blue/basic Fuchsin and analyzed using light microscopy.

Furthermore, image analysis was performed to evaluate the quantity of hard tissue formation in different specimens. Three tissue sections per implant were digitized at low magnification (2.5×), allowing coverage of the entire implant. Using the Leica® Qwin Pro image analysis system (Leica, Wetzlar, Germany), the areas of the ceramic scaffold and the novel bone-like tissue were detected. The outline of the implant area was designed as the region of interest (ROI). Then, with the use of the image analysis software, the surface area of bone-like tissue (BSA) and the ratio of bone-like area (BSA/ROI) were calculated [Castano-Izquierdo et al., 2007].

STATISTICAL ANALYSIS

Statistical significance was evaluated using one-way ANOVA with post hoc Tukey testing, and $P < 0.05$ was considered significant. Error bars represent mean ± standard deviation (SD).

RESULTS

CELL PROLIFERATION AND CELL VIABILITY

With pro-inflammatory cytokine treatment, all three groups (IL-1β, TNF-α, and IL-1β + TNF-α) showed similar cell proliferation profiles (Fig. 1A). When compared to the control group (without cytokine), the DNA results showed significantly faster cell proliferation for 3-day treatment, especially for IL-1β + TNF-α group. However, after that, the cell numbers consistently decreased. To assess cell viability, the mitochondrial activity was measured using a MTT-based assay. The normalized results are presented (Fig. 1B), in which the normalized values are corrected for cell number. Cell viability of all three treatment groups showed continuous decrease from days 7 to 12, and was significantly lower compared to control group ($P < 0.01$).

ALP ACTIVITY

The effects of pro-inflammatory cytokines on the ALP activity are depicted in Figure 1C. DPSCs treated with for 3 days had greater ALP activity than the negative control. Among all three groups, the ALP activity was increased most by IL-1β + TNF-α treatment. Conversely, with prolonged treatment, the ALP activity was depressed by cytokine treatment.

OSTEOCALCIN AND BONE SIALOPROTEIN EXPRESSIONS

The expression of OC and BSP was depicted in Figure 1D,E. Both proteins almost showed similar expression profiles in all three treatment groups. The expression increased significantly at day 3 compared to control group and declined after that. Further, among all three treatment groups, IL-1β + TNF-α group showed higher protein levels compared to only IL-1β or TNF-α group.

REAL-TIME QUANTITATIVE PCR

The mRNA expression levels for Alp, OC, Bsp, Dspp, and Dmp1 were compared among these four groups (Fig. 2). Expression levels of cells from control group at day 3 were set as baseline control (relative expression value, 100%). The expression of the five measured genes always showed similar profiles to each other in all three treatment groups, with expression increasing significantly at day 3 compared to control group and decreasing after that. The most obvious differences were that IL-1β + TNF-α treatment demonstrated most up-regulation of expression of all five measured genes compared to only IL-1β or TNF-α treatment. Conversely, control group showed the overall expression increasing for these genes with time.

SEM AND EDS ANALYSIS

On day 3 with treatment, SEM observation showed that cells had adhered well and spread fully on the surfaces of the HA/TCP ceramic scaffolds. A noticeable amount of mineralized nodules appeared on cell/scaffold complexes. There were no obvious morphological differences between these three treatment groups (Fig. 3). For the control group, an intact cell layer was present without obvious

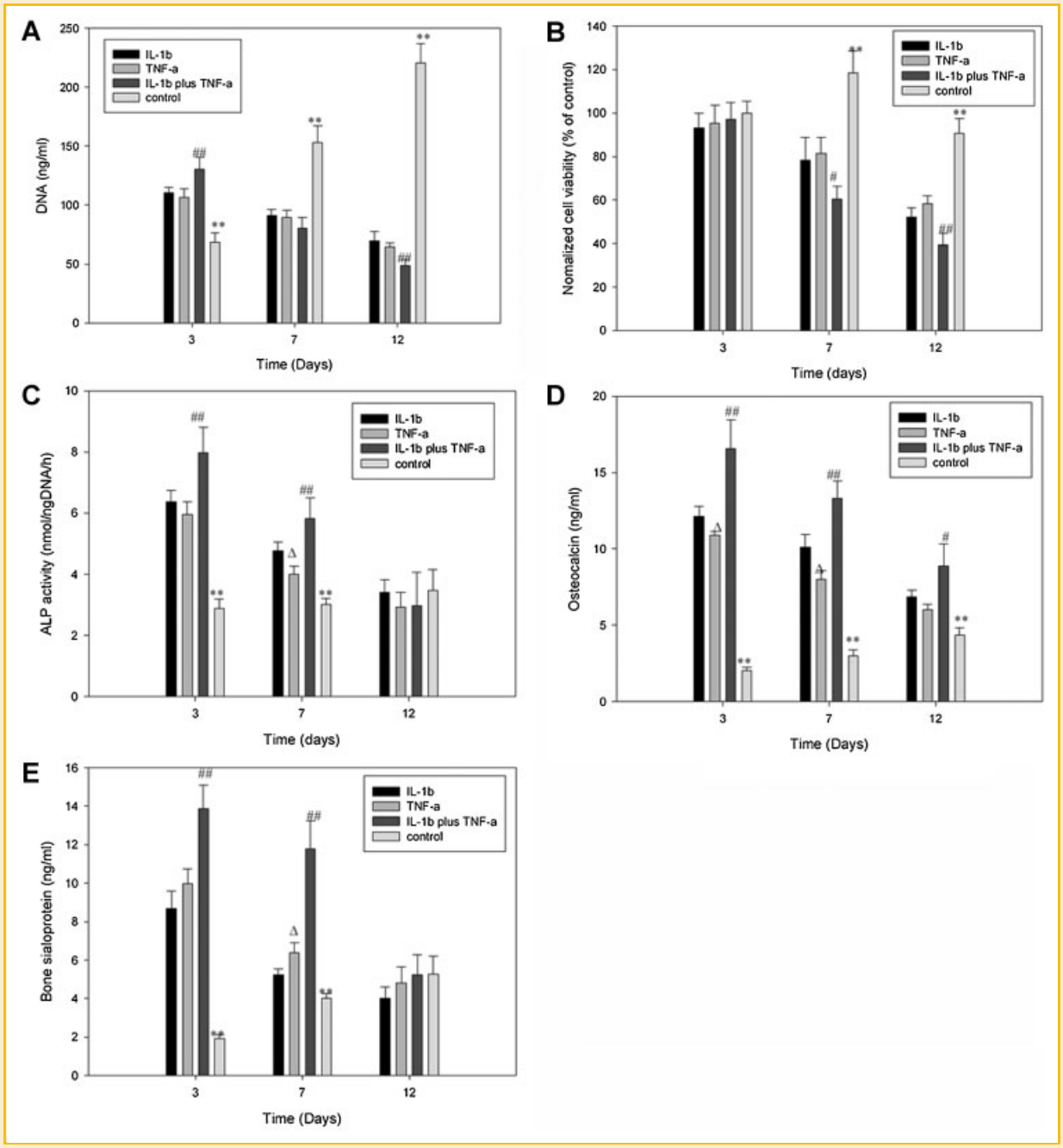

Fig. 1. A: The proliferation characteristics. B: Cell viability analysis. C: ALP activity. D: OC assay. E: BSP assay. The bars represent the mean ± SD (n = 6). Control group compared to IL-1β group, TNF-α group, and IL-1β + TNF-α group: $^{*}P < 0.05$, $^{**}P < 0.01$; IL-1β + TNF-α group compared to IL-1β and TNF-α group: $\#P < 0.05$, $\#\#P < 0.01$; TNF-α group compared to IL-1β group: $\triangle P < 0.05$.

mineralized deposits only showed cells growing on the scaffolds. EDS analysis revealed the presence of calcium and phosphorus in deposits at a Ca:P ratio of 1.6–1.8.

IN VIVO ANALYSIS

All mice appeared to be in good health throughout the test period. There were no symptoms of severe inflammation at the implantation sites. After sacrifice, all implants were retrieved with the surrounding soft tissues intact.

Histological observation was performed under light microscopy. All ceramic implants were encapsulated with a very thin fibrous tissue capsule, closely following the outer perimeter of the scaffold. Inflammatory cells were only sporadically present. Tissue ingrowths were found in all the implants and macro-pores of scaffolds were filled with fibrous tissue.

For 8 weeks of implantation, all the ceramic implants seeded with pro-inflammatory cytokine-treated DPSCs showed obvious hard tissue formation (Fig. 4A–C). Especially, the IL-1β + TNF-α group

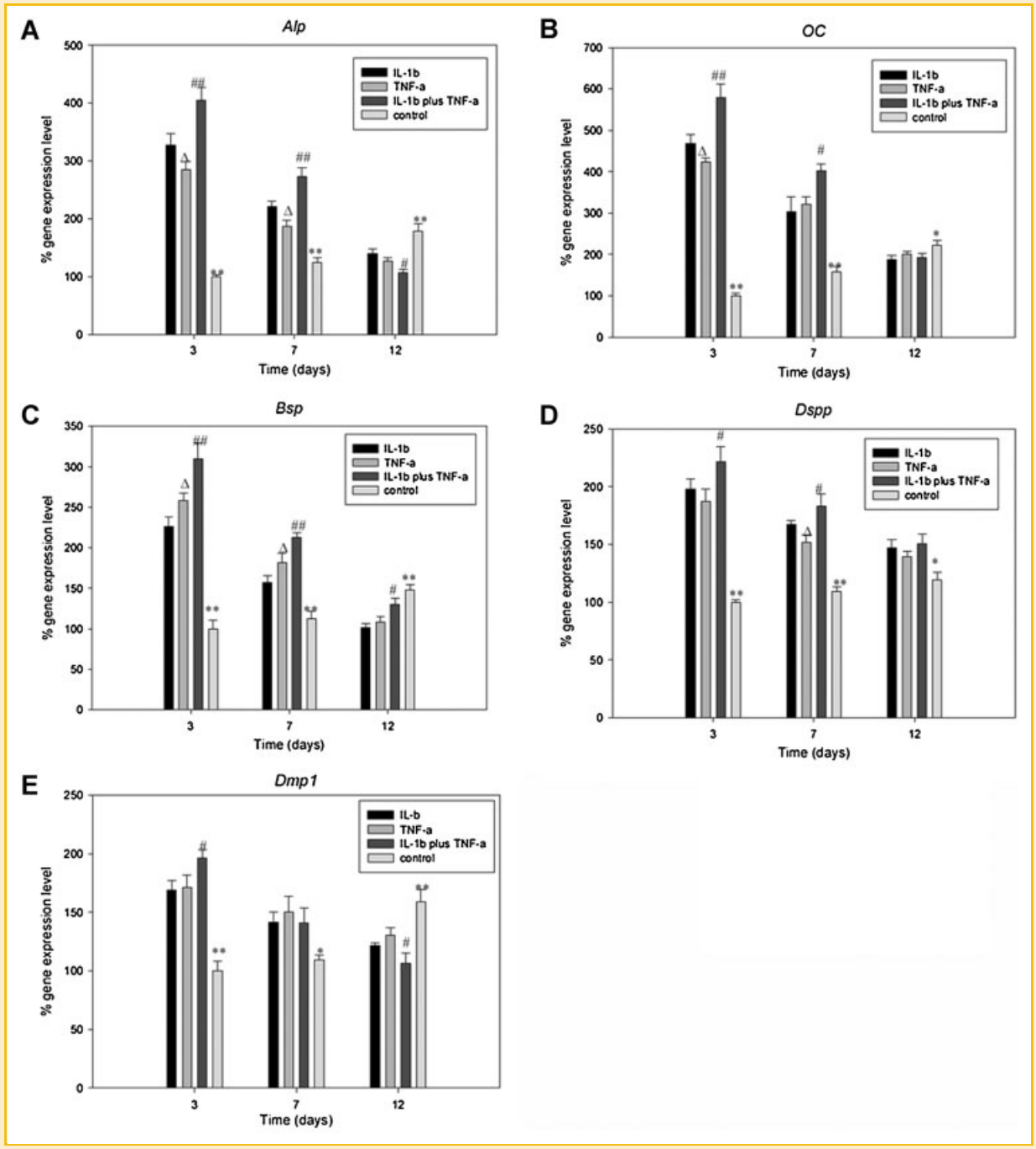

Fig. 2. A: Curves of the mRNA levels of ALP; (B) OC; (C) BSP; (D) DSPP; (E) DMP-1. The bars represent the mean ± SD (n = 3). Control group compared to IL-1β group, TNF-α group, and IL-1β + TNF-α group: $^{*}P < 0.05$, $^{**}P < 0.01$; IL-1β + TNF-α group compared to IL-1β and TNF-α group: $\#P < 0.05$, $\#\#P < 0.01$; TNF-α group compared to IL-1β group: $\triangle P < 0.05$.

upon visual inspection showed more hard tissue formation compared to the other two groups. The newly formed hard tissue was stained deep red by the basic Fuchsin and resided mainly along the surface of the porous ceramic material. At higher magnification (Fig. 5A), distinct concentric lamellae of mineralized matter were observed with entrapped osteocyte-like cells and osteoblast-like cells lying orderly arranged at the surface. Bone marrow-like hematopoietic tissue was also seen together with the newly formed hard tissue. In the samples loaded with non-treated DPSCs, still no specific newly hard tissue formation was found (Fig. 4D). Only an orderly arranged thick fibril-like extracellular matrix (ECM) with multiple-layers of cells was seen, organized parallel to the pore surface (Fig. 5B).

Further, histomorphometry was performed to measure the percentages of hard tissue formation in the various implant groups. With the 8-week implantation periods, hard tissue formation per

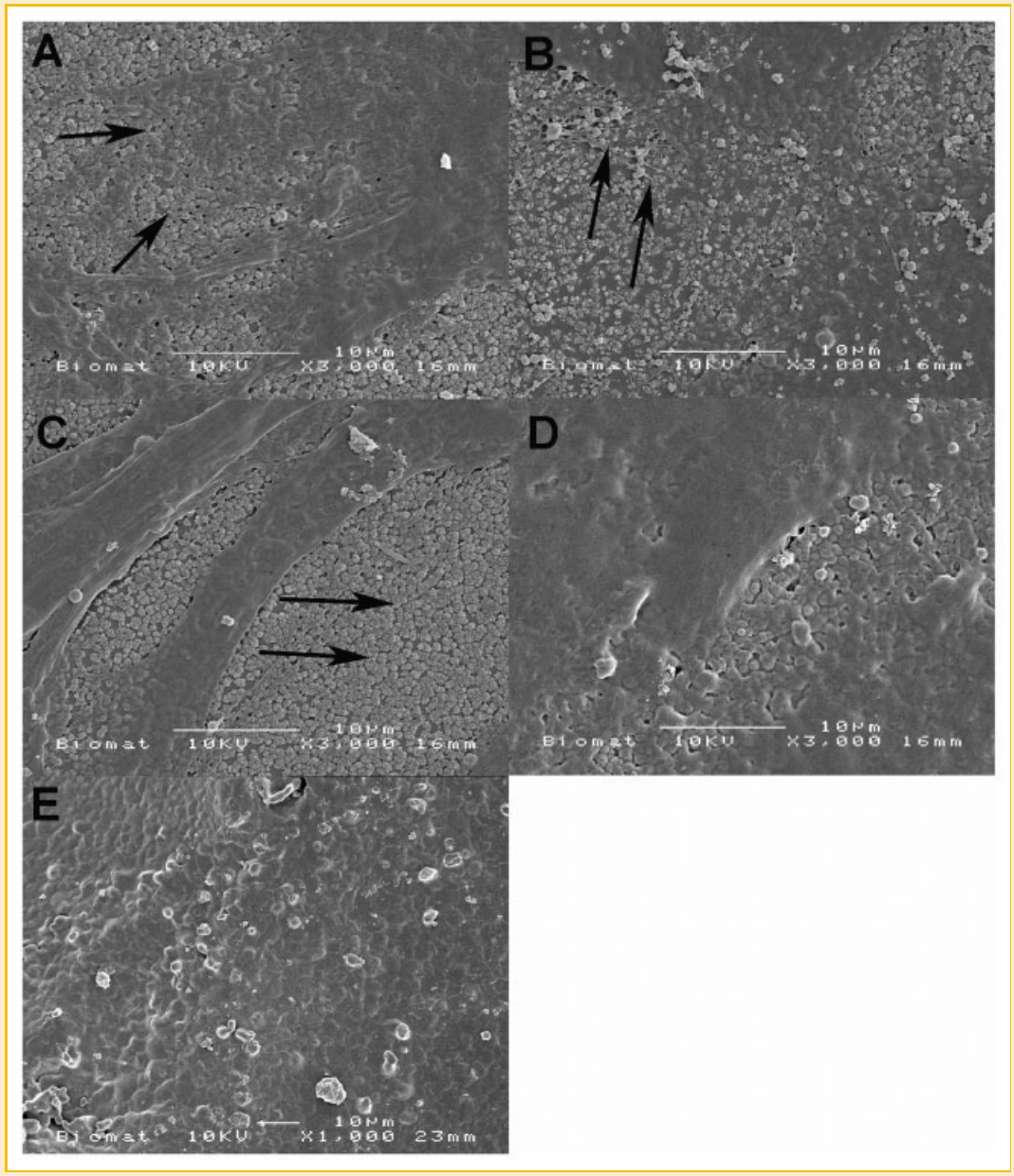

Fig. 3. Scanning electron micrographs with 3-day treatment. A: IL-1β group. B: TNF-α group. C: IL-1β + TNF-α group. D: Control group. E: Empty scaffold. Arrows indicate some of the calcified nodules.

implant was 11.9 ± 3.1% of pore area for IL-1β group, 10.5 ± 1.8% for TNF-α group, and 17.8 ± 2.2% for IL-1β + TNF-α group, respectively. The values revealed more hard tissue formation in IL-1β + TNF-α group ($P < 0.05$). Moreover, all three groups above showed significantly more bone-like hard tissue formation, compared to the non-treated group (3.1 ± 0.8%, respectively; $P < 0.01$).

DISCUSSION

DPSCs, when as seed cells applied for tissue engineering and dental tissue repair, mostly are exposed to unfavorable inflammation conditions. This study examined the possible behaviors of DPSCs when exposed to inflammation conditions. As we know, this is the first report that pro-inflammatory cytokines induce mineralization potential of DPSCs in vitro and in vivo.

Pro-inflammatory cytokines can be rapidly produced by tissue cells with in tenths of minutes when contacting with pathogens, and are able to trigger potent inflammatory responses [Keller et al., 2010]. Among these cytokines, TNF-α and IL-1β have a critical role in the regulation of the many outputs of inflammation [Tincani et al., 2007]. TNF-α and IL-1β are not or barely expressed in healthy pulps but are up-regulated in inflamed pulps. Cell origin of TNF-α is unknown, but IL-1β was shown to be produced by macrophages [Keller et al., 2010].

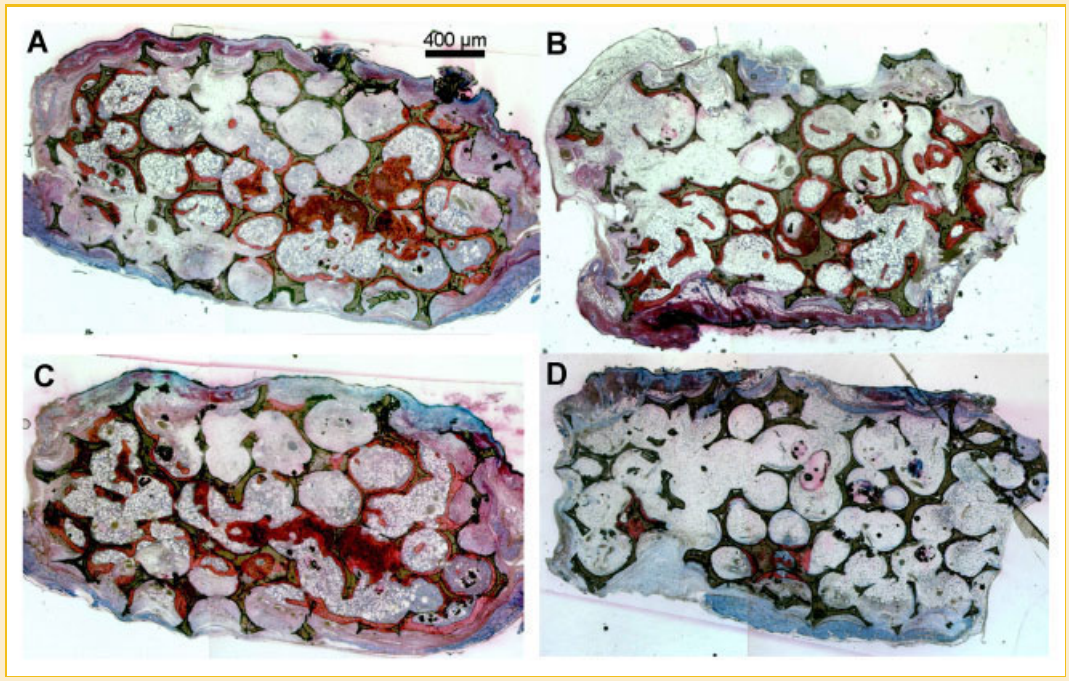

Fig. 4. General histological overview of the implants of 8 weeks (methylene blue basic/Fuchsin staining). A: IL-1β group. B: TNF-α group. C: IL-1β + TNF-α group. D: Control group. Scale bar is 400 μm (original magnification 2.5×).

In our study, the pro-inflammatory cytokine treatment significantly provoked DPSCs proliferation during 3 days, and conversely inhibited after that. This change is in accordance with the results of cell viability. Previous study showed that a short treatment of TNF-α or IL-1β can promote cell DNA synthesis [Lange et al., 2010], while the long treatment of pro-inflammatory cytokines may induce cell apoptosis.

ALP is a prerequisite for the differentiation of dental pulp in vivo. Dental pulp tissue has high activity of ALP during dentinogenesis, which might be an important component of the repair mechanism and healing after pulp injury. Previous study have demonstrated that IL-1 induced ALP activity and produced mineralization-related proteins in short-term culture of dental pulp cells, while inhibited those effects during the long-term culture [Min et al., 2006]. In our study, we also observed that ALP activity increased markedly in IL-1β- or/and TNF-α-treated DPSCs at 3 days and decreased with prolonged culture. This fact may indicate that pro-inflammatory cytokines affect ALP activity during early pulpitis. Previous findings also showed that ALP activity was very high in reversible pulpitis compared to normal healthy pulp or irreversible pulpitis [Spoto et al., 2001].

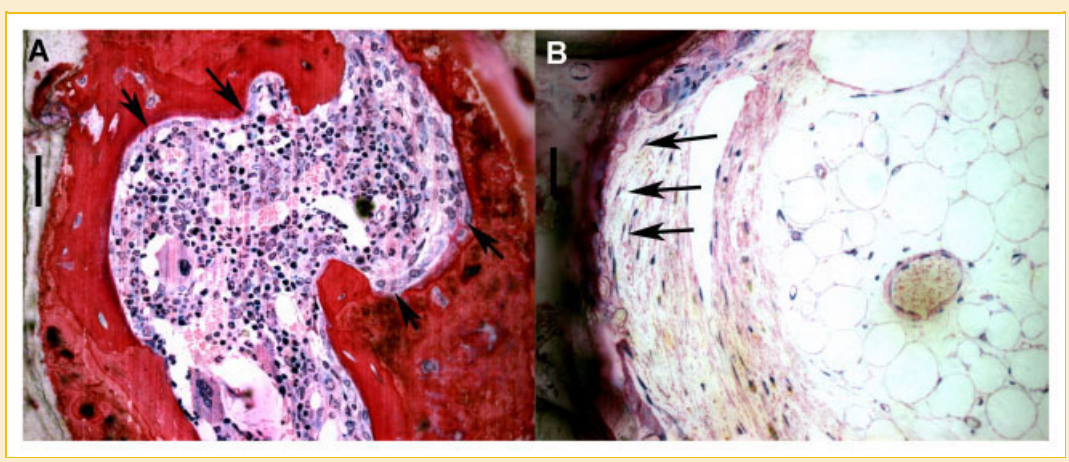

Fig. 5. Histology at a high magnification (methylene blue basic/Fuchsin staining). A: IL-1β + TNF-α group at 8 weeks. Note the orderly array of osteoblast-like cells on the surface of the bone-like matrix (arrow; original magnification 40×), bar size is 25 μm. B: Control group at 8 weeks. Note the multiple layers of cells orderly arranged along the pore surface (arrow), however lacking the aspect of bone-like tissue formation (original magnification 40×), bar size is 25 μm.

Some non-collagenous proteins in dentin or bone include OC and BSP. Besides, DSPP is the major part of non-collagenous proteins in dentin, and plays a crucial role during dentin mineralization [Feng et al., 1998]. DMP-1 is another specific protein in dentin, and is a candidate gene for dentinogenesis imperfecta [MacDougall et al., 1996]. Although the different genes showed different mRNA expression levels, their expression was more or less increased after a short treatment of IL-1β and TNF-α. The protein assay for OC and BSP also confirmed the similar expression trend. This change was comparable to that of DPSCs under osteogenic culture [Zhang et al., 2005; Yang et al., 2007ac]. The expression of these markers offers strong evidence that DPSCs differentiate into odontoblasts by the short presence of pro-inflammatory cytokines. The decrease of these gene expressions for 7 and 12 days, probably a result of cell apoptosis caused by irreversible inflammation with long-term exposure of pro-inflammatory cytokines, may also indicate an important role of IL-1β and TNF-α for the mineralization processes in the early inflammatory condition of pulp tissue. Moreover, previous study reported that TNF-α alone treatment inhibited BSP expression of pulp cells [Min et al., 2006]. However, in this study, we observed converse results. These differences may partially due to the different cell type used. Furthermore, the combination of IL-1β and TNF-α also showed increased effects, whatever for up-regulation or down-regulation of these gene expressions.

A porous calcium phosphate ceramic carrier was selected as scaffold material. Such ceramic also is used in other studies for hard tissue regeneration, because of its bone-compatible character and the ability to support the differentiation of bone and dentin-forming cells [Hartman et al., 2005; Yang et al., 2009ab]. Results of the in vitro part of our current study indicated that the DPSCs seeded on the HA/TCP materials were capable of producing a mineralized ECM with 3-day pro-inflammatory cytokine treatment. These results were analogous to these of cells under the influence of a standard osteogenic medium [Zhang et al., 2005; Yang et al., 2007c].

Our previous study indicated that DPSC–HA/TCP complexes precultured with medium containing dexamethasone elicited $21.9 \pm 4.1\%$ of new hard tissue in the pore area after 12-week implantation [Yang et al., 2009a]. Although less than that, our current study also demonstrated $11.9 \pm 3.1\%$, $10.5 \pm 1.8\%$, and $17.8 \pm 2.2\%$ of new hard tissue in pore area for IL-1β, TNF-α group, and IL-1β + TNF-α groups, respectively. These cytokine-treated DPSCs showed the potential of hard tissue regeneration in vivo, which can regenerate bone-like hard tissue formation in subcutaneous implantation model, even no hard tissue promoting supplements (such as dexamethasone) were used. The results were consistent with our in vitro results, which indicated that a short-term (3 days) exposure of pro-inflammation cytokines induce the expressions of mineralization-relative factors in DPSCs. Although the mechanism is not so clear, we presume the mineralization response of DPSCs to the pro-inflammatory cytokines may help host defense and tissue repair in early-inflamed pulp tissues.

In summary, this study demonstrated that pro-inflammatory cytokine IL-1β or/and TNF-α induced ALP activity and mineralization-related gene expressions in DPSCs in vitro with a short-term culture, while inhibited those effects during the long-term culture. Further, with a short-term treatment in vitro, DPSCs showed an obvious behavior for mineralization tissue formation, even without externally added substitutes (i.e., dexamethasone). These data suggest that IL-1β and TNF-α produced in the early inflammatory reaction may induce the mineralization of DPSCs, which may be an important mechanism for host defense and tissue repair.

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