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# Expression of vascular endothelial growth factor and its receptors after mandibular distraction osteogenesis

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*Abstract.* During distraction osteogenesis, angiogenic activity is essential for new bone formation. This study examined the expression of vascular endothelial growth factor (VEGF) and two of its receptors, Flt-1 (VEGFR-1) and Flk-1 (VEGFR-2), in cellular components after mandibular distraction osteogenesis. Unilateral mandibular distraction (0.5 mm twice per day for 10 days) was performed in six mongrel dogs. Two animals each were killed on days 7, 14 and 28 after completion of distraction. The distracted mandibular segments and contralateral undistracted control segments were harvested and processed for immunohistochemical examination. Seven days after distraction, there was a significant increase in the expression levels of VEGF and its receptors in the osteoblasts, osteocytes and immature fibroblast-like cells compared to control specimens. These levels were maintained for 14 days after distraction in the osteoblasts and fibroblast-like cells. Twenty-eight days after distraction, VEGF and VEGFR-1 were expressed only moderately/weakly in the osteoblasts, and no VEGFR-2 expression was detected in the cellular component of the distracted bone. Throughout the observation period, VEGFR-1 expression was stronger than that of VEGFR-2. The expression patterns of VEGF and its receptors suggest that it plays an important role in osteogenesis, and that osteoblasts and immature fibroblast-like cells of the distracted bone may have an autocrine growth effect during distraction osteogenesis.

Key words: distraction osteogenesis; vascular endothelial growth factor; Flt-1; Flk-1; autocrine growth effect.

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Distraction osteogenesis (DO) is a useful method for treating cases demanding the generation of new bone. Despite the fact that DO is used in various fields, there have been few studies[2,7,27,32] undertaken at the cellular and molecular level. These studies suggest that some growth factors, such as transforming growth factor-beta (TGF-β), insulin-like growth factor-I (IGF-I), bone morphogenetic proteins (BMPs) and basic fibroblast growth factor (bFGF), play important roles in new bone formation after DO. In addition, there is increasing interest in the relationship between osteogenesis and angiogenesis during DO[3,6].

Bone formation is closely related to the formation of blood vessels. Several studies[10,11] have shown that osteoblasts and osteoblast-like cells can produce vascular endothelial growth factor (VEGF), and

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that this process may be tightly regulated by osteogenic factors, including TGF-β and IGF-I. It is also the case that angiogenic factors, such as VEGF and bFGF, can amplify osteogenic activity[15]. Of the various angiogenic factors, VEGF is perhaps the most critical driver of vascular formation during angiogenesis and vasculogenesis, and has been demonstrated to play a crucial role during osteogenesis[12].

VEGF is characterized as a heparin-binding angiogenic growth factor that displays a high specificity for endothelial cells, and is structurally related to platelet-derived growth factor[19]. Several VEGF receptors (VEGFRs) belonging to the tyrosine-kinase receptor family have been identified and cloned, e.g. VEGFR-1 (*fms*-like tyrosine kinase receptor 1, Flt-1), VEGFR-2 (fetal liver kinase 1, Flk-1) and VEGFR-3 (Flt-4). VEGFR-2 appears to mediate the differentiation and proliferation of endothelial cells, and the activation of VEGFR-2 by VEGF results in a mitogenic response. VEGFR-1 appears to play an important role in vascular maintenance as well as in the recruitment of endothelial precursor cells during vasculogenesis. The activation of VEGFR-1 by VEGF appears to induce cell migration. VEGFR-3 is believed to play a role in the development of lymphatic as well as blood vessels[19]. It is unclear whether the roles of the VEGFRs in osteogenic cells are similar to those in endothelial cells.

Masood et al.[16] reported the concurrent expression of VEGF and VEGFRs in a number of tumour cells, and suggested that VEGF here functioned as an autocrine growth factor. Several other studies[5,8,17,18] have also demonstrated the simultaneous expressions of VEGF and VEGFRs during osteoblast differentiation. In this study was examined the autocrine growth activity of the cellular components of a distracted bone after mandibular DO, with regard to the expression of VEGF and its receptors.

## Materials and methods

### Animal model and surgical protocol

Six mongrel dogs, aged between 1 and 2 years and weighing approximately 10 kg, were used in this study. The animal model and surgical protocol were as described previously[21]. All experimentation was performed after gaining authorization from the Animal Center for Medical Experimentation at Gyeongsang National University.

The animals were anaesthetized by an intravenous injection of a mixture of 10 mg/kg of ketamine (Ketalar®, Yuhan Corp., Korea) and 2.0 mg/kg of 2% xylazine (Rompun®, Bayer Korea). The surgical fields were sterilized with betadine solution and 2% lidocaine HCl containing 1:100,000 epinephrine was then injected into the right submandibular skin. After sequential dissection of the submandible, buccal and lingual corticotomies were conducted between the 3rd and 4th premolars, or between the 4th premolar and the 1st molar. The intraoral mandibular distractor (Leibinger, Germany) was then positioned on the buccal cortical bone, after the mandible had been carefully fractured in a linear manner. The distractor rod was exposed by perforating the retromandibular skin. The wound was closed in two layers with 3-0 Vicryl for the platysma and 3-0 nylon for skin. First generated cephalosporin (20 mg/kg; Cefazolin®, Yuhan Corp.) was injected intramuscularly twice a day for 5 days after surgery. After a 5-day latency period, the mandible was distracted for 10 days at a rate of 1.0 mm/day in two increments per day.

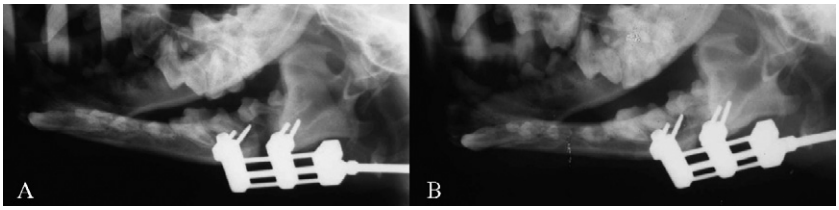


*Fig. 1.* Radiographs demonstrating the distraction and healing of the mandible. (A) Immediately after completing the distraction, showing a distraction gap. (B) Twenty-eight days after the distraction, note the presence of bone consolidation within the gap.

### Specimen preparation

After the administration of general anaesthesia, two animals each were killed by KCl injection at 7, 14 and 28 days after completion of distraction. The right distracted mandibles (the distraction group) and the left undistracted mandible (the control group) were then harvested *en bloc* using an identical procedure. The harvested bony tissue specimens were fixed in 10% neutral buffered formalin for 24 h, and then decalcified in 5% nitric acid for 1 week. After tissue processing,

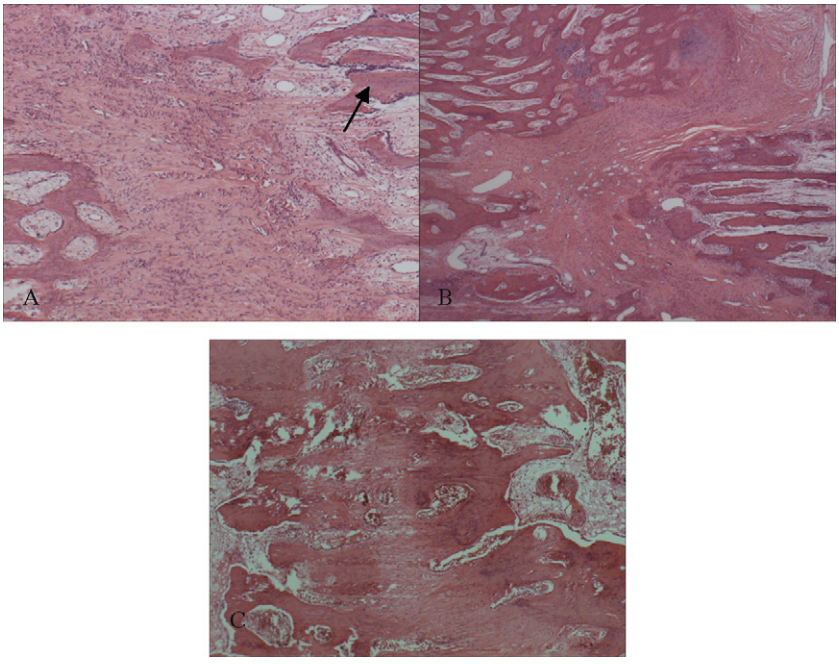


*Fig. 2.* Hematoxylin and eosin-stained section. (A) Seven days after completing the distraction. Numerous immature fibroblast-like cells were observed in the gap interzone. At this time, many osteoblasts lining the immature trabecular bone were also found (arrow) (original magnification ×40). (B) Fourteen days after the distraction. A fibrous interzone was also present at this time (original magnification ×20). (C) Twenty-eight days after the distraction. The fibrous interzone was almost filled with newly formed bone (original magnification ×40).

the specimens were embedded into paraffin blocks. The paraffin blocks were cut into 4 μm sections, and the sections were mounted on silane-coated slides in order to minimize tissue loss during the staining process. The sliced sections were maintained at room temperature for 12 h, and then deparaffinized and hydrated.

### Immunohistochemical staining

Immunostaining was performed using an automated immunostainer (Ventana, Biotek Systems, Tucson, AZ, USA). A 1:500 dilution of primary rabbit polyclonal antihuman VEGF (sc-507, Santa Cruz, CA, USA) was used to visualize the VEGF expression, and a 1:100 dilution of primary rabbit polyclonal antihuman Flt-1 (Neomarkers, CA, USA) and a 1:100 dilution of primary rabbit polyclonal antihuman Flk-1 (Neomarkers) were used to observe the expressions of the VEGFRs. The sections immunostained for VEGF and VEGFRs were subjected to antigen retrieval by treating them with 0.1% trypsin solution (S2012, Dako, Denmark) at 37 °C for 20 min. The antigen-retrieved sections were then treated with hydrogen peroxide in order to block endogenous peroxidase activity. The sections were then reacted with primary antibodies against VEGF and VEGFRs at 37 °C for 32 min. The sections were then treated with a biotinylated polyvalent secondary antibody solution, incubated with a horseradish peroxidase-conjugated avidin–biotin complex, and treated with 3,3-diaminobenzidine and hydrogen peroxide. Finally, the nuclei were counterstained with hematoxylin.

### Histological evaluation

The immunohistochemical expression was assessed using optical microscopy. Two experienced pathologists, who were blinded to the staining and stage details, evaluated the immunohistochemical staining patterns. A minimum of three sections per animal were evaluated at each time point for each protein analysed. The slices were also examined for any antibody deposition in the cellular components, including osteoblasts, osteocytes, and fibroblast-like cells. According to the methods reported by Tavakoli et al.[27], Hu et al.[12], and Knabe et al.[14], the immunostaining intensity was graded as +++, ++, + or − for strong, moderate, weak and negative staining, respectively. A grade of ± was used to represent focal or questionable weak staining.

## Results

The DO proceeded smoothly in all of the animals with no surgical infections or failures. The change in the length of the mandible was determined from the change in the distance between the proximal and distal pins, which was measured immediately after placing the distractor and prior to killing. The mandible was lengthened by a mean of 8.8 mm ± 0.8 mm in six dogs, which is a similar amount to that reported in a previous paper[21]. Twenty-eight days after completing the distraction, new bone formation at the distracted mandible was observed by radiography (Fig. 1).

### Histological examination

Using optical microscopy with hematoxylin and eosin staining, the distracted zones were found to have completely united during the consolidation period, predominantly by intramembranous ossification. An immature fibrous interzone was observed at 7 and 14 days after the distraction. At these times, numerous osteoblasts lining the immature trabecular

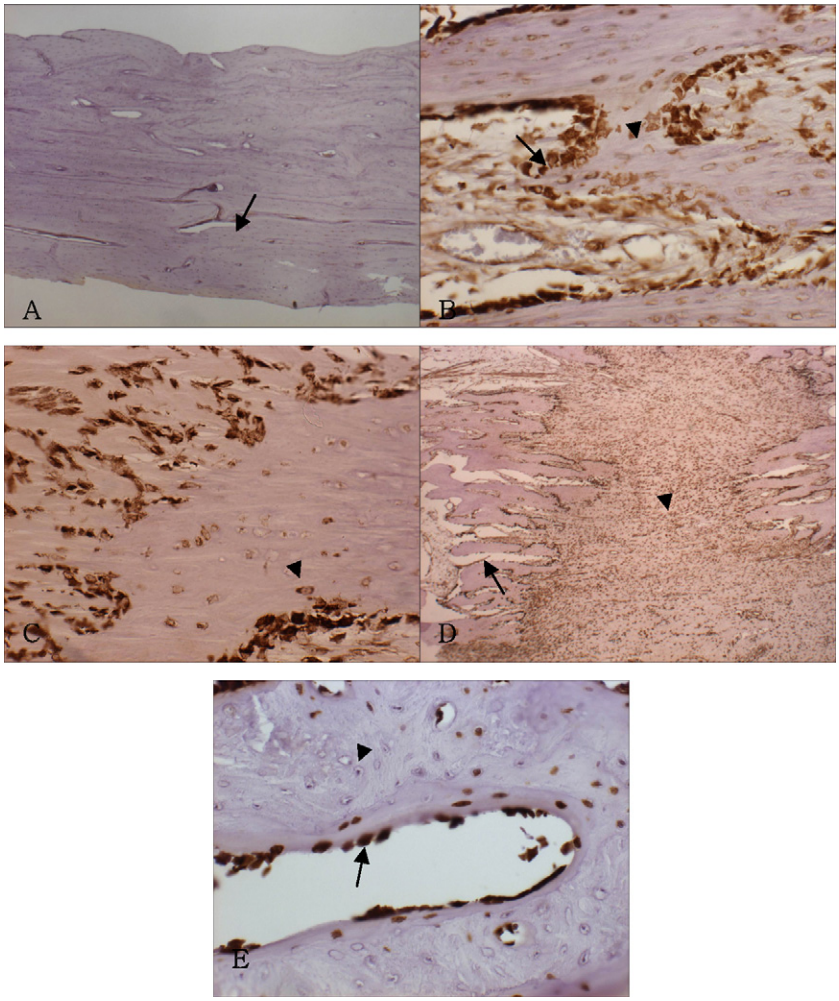


*Fig. 3*. VEGF expression in cellular components during osteogenesis. (A) VEGF was not expressed in the control specimen except in vascular tissues (arrow) (original magnification ×40). (B) Seven days after distraction. Strong VEGF signals were detected in the osteoblasts (arrow) lining the immature trabecular bone. At this time, elevated VEGF signals were also detected in osteocytes (arrowhead), fibroblast-like cells, and in endothelial cells (original magnification ×200). (C) This increase in VEGF expression persisted in the lining osteoblasts and immature fibroblast-like cells for 14 days after distraction. At this time, VEGF was expressed weakly only in the osteocytes, which were located near the osteoblasts (arrowhead) (original magnification ×200). (D) Immature gap at 14 days after distraction; note the positive staining of fibroblast-like cells (arrowhead) and osteoblasts (arrow) lining the newly generated bone (original magnification ×40). (E) Twenty-eight days after distraction, positive staining was found in osteoblasts (arrow) lining the trabecular bone, but the osteocytes were almost negative (arrowhead) (original magnification ×200).

bone were also detected. Twenty-eight days after the distraction, the distracted interzone was filled with mature bone (Fig. 2).

### Immunohistochemical evaluation

VEGF was not detected in control specimens except in the endothelial cells. Seven days after completing the distraction, the VEGF expression level was elevated in all cellular components of distracted specimens. At this time, strong VEGF expression was observed in the osteoblasts and fibroblast-like cells, whereas its expression was moderate in the osteocytes. Even at 14 and 28 days after distraction, the VEGF expression level was strong to moderate in the osteoblasts, but it decreased in the osteocytes and fibroblast-like cells between 14 and 28 days (Fig. 3).

There was no Flt-1 and Flk-1 expression in the undistracted control specimens. Strong or moderate Flt-1 expression was observed in the osteoblasts and fibroblast-like cells at 7 days after distraction, and moderate expression was observed in the osteoblasts until 14 days after distraction. Flt-1 expression weakened or disappeared in the other cellular components between 14 and 28 days (Fig. 4). Flk-1 expression increased to a moderate level in the osteoblasts at 7 days after distraction but was weak at 14 days and negative at 28 days. Seven days after distraction, Flk-1 was weakly expressed in the osteocytes but was not detected in the fibroblast-like cells (Fig. 5). Flk-1 expression was weak overall compared with that of Flt-1. Table 1 summarizes the patterns of VEGF and VEGFR expression in the cellular components after mandibular DO.

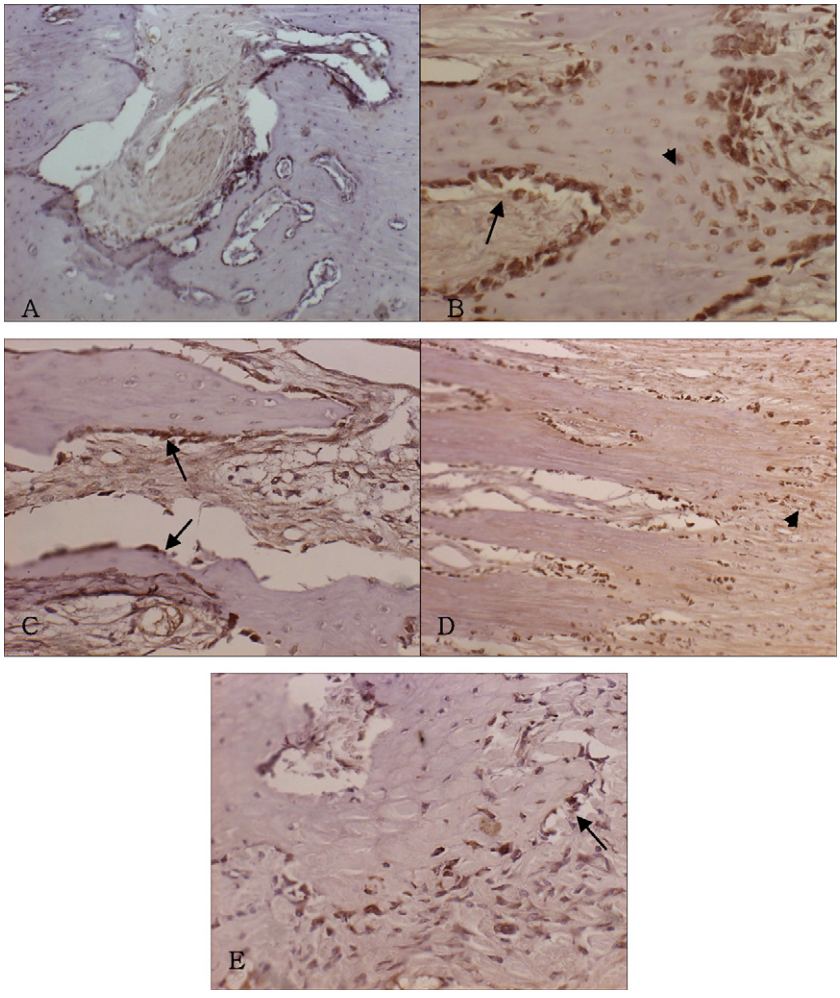


*Fig. 4*. Immunostaining of Flt-1 (VEGFR-1) in distracted bone. (A) Note the negative staining of the cellular components in the undistracted control specimen (original magnification ×100). (B) Flt-1 was strongly expressed in the osteoblasts (arrow) and weakly expressed in the osteocytes (arrowhead) at 7 days after distraction (original magnification ×200). (C) At 14 days after distraction, increased staining was observed only in the lining osteoblasts (arrows) (original magnification ×200). (D) Fourteen days after distraction; note the weak staining in the immature fibroblast-like cells (arrowhead) (original magnification ×100). (E) Twenty-eight days after distraction, weak Flt-1 staining was observed in the osteoblasts (arrow) (original magnification ×200).

## Discussion

Several animal models have been used to study mandibular DO, such as sheep[7,27], goats[12], rats[6,31], dogs[13,21], monkeys[30] and pigs[28,32,33], and a recent study[14] reported the expression of several growth factors in a human distracted mandible. Some authors[28,33] have argued that the porcine model is relatively inexpensive and easy to handle, and its mandibular size and shape are similar to human. The data from a sheep model of mandibular DO have also been reported in detail[7,14,27]. Dogs are also easy to handle and sufficiently large to survive the surgical procedure[13]. The dog model was the first used to examine mandibular DO[13] and the surgical protocol and results have been relatively well described. A commercial mandibular distractor can be placed in a dog's mandible without the need for adjustment.

In this study, the immunohistochemical expression of the cellular components was examined semi-quantitatively. In previous reports[12,14,27] this method was used to detect growth factors in cellular and matrix components after DO. Knabe et al.[14] reported that semi-quantitative analysis is an excellent tool because, within statistical boundaries, experienced investigators blinded to the type of staining obtained the same results. It is also believed that semi-quantitative analysis is a more useful method when performed on cellular components.

*Table 1*. Semi-quantitative analysis of staining of VEGF and VEGFRs in cellular components after mandibular DO

| | Osteoblasts | | | Osteocytes | | | Fibroblast-like cells | | |
|---|---|---|---|---|---|---|---|---|---|
| | VEGF | Flt-1 | Flk-1 | VEGF | Flt-1 | Flk-1 | VEGF | Flt-1 | Flk-1 |
| Control | – | – | – | – | – | – | – | – | – |
| 7 days | +++ | +++ | ++ | ++ | + | + | +++ | ++ | – |
| 14 days | +++ | ++ | + | + | – | – | ++ | + | – |
| 28 days | ++ | + | – | ± | – | – | ± | ± | – |

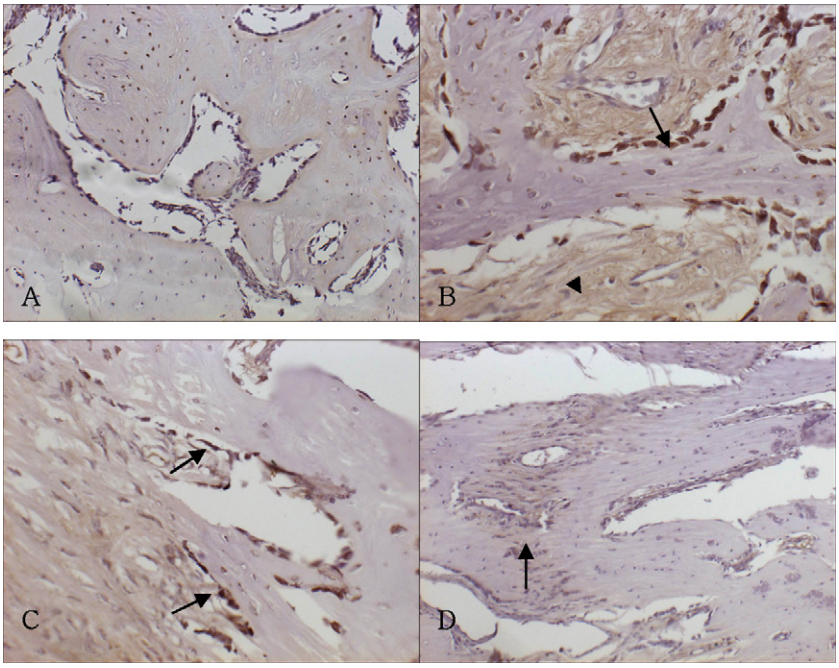


*Fig. 5.* Immunostaining of Flk-1 (VEGFR-2) in distracted bone. (A) Control specimen, showing no staining of cellular components (original magnification ×100). (B) Seven days after distraction. Flk-1 was moderately expressed in the osteoblasts (arrow) lining the immature trabecular bone. Flk-1 was not observed in the fibroblast-like cells (arrowhead) at this time (original magnification ×200). (C) Fourteen days after distraction. Weak Flk-1 staining was detected only in the osteoblast lining the trabecular bone (arrows) (original magnification ×200). (D) Twenty-eight days after distraction; note the negative expression in the cellular components of the distracted zone. The arrow indicates newly formed bone in the distracted interzone (original magnification ×100).

Many researchers have reported that angiogenesis and vasculogenesis are important factors in osteogenesis. Aronson[1] observed an almost 10-fold increase in blood flow in osteotomized bone segments compared with non-osteotomized controls. After distraction, increased neovascularization during osteogenesis has been observed by scanning electron microscopy[4] and angiography[23]. The expression of many osteogenic and angiogenic factors has been detected during DO[20,27,31]. Of these factors, VEGF is probably the most important for angiogenesis and osteogenesis.

Several studies[2,7,25,27] have demonstrated that the VEGF secreted by osteogenic cells may be regulated by a number of inflammatory cytokines as well as by hypoxia in a similar manner to that observed in endothelial cells. Steinbrech et al.[25] reported that VEGF expression in osteoblasts is modulated by a hypoxia response mechanism. They observed a three-fold increase in VEGF levels over a 24-h period after exposing osteoblast-like cells to hypoxic conditions. Spector et al.[24] reported that the VEGF expression level in osteoblasts is reduced in specific environments, such as those with an acid pH or elevated lactate levels. They suggested that the extracellular microenvironment and hypoxia control the VEGF expression level of osteoblasts.

The VEGF levels are increased when new bone formation occurs due to fracture or distraction. During fracture repair, the VEGF levels in a hematoma increase almost 100-fold and the VEGF serum levels increase five-fold[26]. The precise effect of VEGF on osteoblastic cells is not completely understood. Several studies have shown that VEGF stimulates the proliferation, migration and differentiation of osteoblasts[5,8,17,18]. In contrast, Furumatsu et al.[9] demonstrated that VEGF had no direct effect on the proliferation of osteoblastic cells and Villars et al.[29] reported that VEGF had no proliferative effect on osteoblast progenitors derived from human bone marrow stromal cells.

In this study, VEGF was strongly expressed in the cellular components of a distracted callus at 7 days after completion of distraction. This result is in agreement with those of other studies[12,31], which reported that VEGF expression is highest during the early consolidation period of DO. Richard et al.[22] also reported that bone formation activity increased the most 6 days after distraction, when the VEGF expression level was at its highest. After distraction, it is believed that the VEGF level increases during the early stage of new bone formation and decreases during the later stage of osteogenesis. This finding partially agrees with the claim that during the distraction process angiogenesis occurs first, followed by osteogenesis after new vessel formation[20].

Of the various VEGF receptors, VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1) are known to participate directly in angiogenesis and vasculogenesis. In endothelial cells, VEGFR-1 is largely involved in cell migration and vascular maintenance, rather than cell proliferation, whereas VEGFR-two participates directly in cell mitogenesis and proliferation[19], but their precise roles in osteogenic cells are not completely understood. In this study, it was found that VEGFR-1 expression was higher than VEGFR-2 expression in osteoblasts and fibroblast-like cells throughout the observation period. This is probably related to the different roles of these two receptors in osteogenic cells. More VEGFR-2 might be produced during the distractor activation period or early consolidation period, and it probably participates in the mitogenesis and proliferation of endothelial cells. VEGFR-1 is probably produced during the middle or late consolidation period because it participates in cellular maintenance. Observation during the distractor activation period or immediately after completing distraction would probably have shown a high VEGFR-2 expression level.

Masood et al.[16] reported the co-expression of VEGF and VEGFRs in some tumour cells and suggested that VEGF is an autocrine growth factor in these cells. Similarly, Decker et al.[5] observed the increased expression of VEGF, VEGFR-1, and VEGFR-2 during mouse preosteoblast-like cell proliferation. They suggested that osteoblast-derived VEGFs might act as paracrine factors that modulate endothelial and osteoblast function, and act as autocrine factors modulating osteoblast differentiation. Recently, Mayer et al.[17] observed increased VEGF-A and VEGFR-1 expression during osteoblast differentiation of human mesenchymal stem cells derived from trabecular bone, and concluded that VEGF-A acts as an autocrine factor for osteoblast differentiation. In contrast, Furumatsu et al.[9] observed VEGF production but not VEGFR-1 or VEGFR-2 expression during the differentiation of human mesenchymal stem cells to osteoblasts. They concluded that

osteoblastic cells might not have an autocrine growth effect.

The results of this study show higher VEGFR-1 and VEGFR-2 expression levels in addition to VEGF expression in the osteoblasts lining immature trabecular bone as well as in fibroblast-like cells in the fibrous interzone during the early consolidation period. This suggests that osteogenic cells have an autocrine effect during new bone formation after distraction. Warren et al.[31] reported that DO is affected by mechanical stimulation, which can induce endogenous VEGF that may regulate angiogenesis and osteogenesis[31]. It is believed that the mechanical stimulation associated with distraction can induce endogenous VEGFRs as well as VEGF in cellular components. This suggests that osteoblasts and immature fibroblast-like cells have an autocrine growth effect during DO.

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