Effect of IGF-I and PDGF administered *in vivo* on the expression of osteoblast-related genes in old rats

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Abstract

In order to establish the cellular basis for using growth factors as possible therapeutic agents for the age-dependent deficit in bone formation activity, we examined the individual and combined effects of IGF-I and/or platelet-derived growth factor (PDGF) on the gene expression of osteoblast-related markers in male rats.

The expression of osteoblast markers was examined in the femurs of adult and old rats following marrow ablation, which amplifies gene expression activity. The mRNA levels of collagen_{α1} (I) (COLI), alkaline phosphatase (AP), osteopontin (OP) and osteocalcin (OC) were significantly lower in the old as compared with the adult rats. To determine whether growth factors can abolish the age-related deficits in mRNA expression in old bone, PDGF and/or IGF-I were infused directly into the right femur for 5 days following marrow ablation. The contralateral femur was infused with vehicle only and used as a control. PDGF stimulated the expression of OP mRNA in both adult and old rats, whereas COLI, AP and OC mRNAs were not affected. IGF-I infusion did not have a significant effect on mRNA expression in adult rats. In contrast, treatment with IGF-I significantly enhanced the

mRNA levels of COLI, AP and OP in old rats. To examine whether the combination of both factors could affect the expression of osteoblast markers synergistically, PDGF and IGF-I were infused together. In adult bones, the combined treatment with PDGF and IGF-I caused a slight increase in the level of OP gene expression but no change in AP, OC or COLI genes. Although neither IGF-I nor PDGF alone was effective in stimulating the expression of OC, the combined treatment in old bones enhanced OC expression significantly. The expression of COLI, AP and OP was also stimulated, but the stimulation was no different from that of IGF-I alone. In PDGF plus IGF-I treatment with a high dose, no dose-response effects were observed.

Within the limits of the present study, it is suggested that IGF-I and, to a much lesser extent, PDGF may partially restore the deficit in the expression of osteoblast markers in old bones, and that the combination of both factors is slightly better than IGF-I alone in stimulating OC expression.

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Introduction

The loss of bone mass in senescence can be attributed to an impairment of bone formation which is regulated by bone-seeking hormones or growth factors, but the underlying cause of reduced bone formation activity in aging is still unclear (Christiansen 1992). It is widely accepted that estrogen has an important protective effect on bone and that the loss of this protection after menopause contributes to the pathogenesis of postmenopausal osteoporosis. Although this protective effect of estrogen is thought to be largely mediated by the inhibition of bone resorption, recent evidence suggests that stimulation of osteoblast activity may also be involved (Samuels *et al.* 1999, 2000).

Bone growth factors are a class of mediators that regulate the proliferation and differentiation of osteoblasts during the bone formation process. Many studies that examine the effects of growth factors on bone formation suggest that growth factors can be used potentially as therapeutic agents in the treatment of bone loss associated with aging.

Platelet-derived growth factor (PDGF) is one of the biological mediators of bone formation (Howes *et al.* 1988, Centrella *et al.* 1989, Pfeilschifter *et al.* 1990). Earlier studies have shown that PDGF enhances DNA and collagen synthesis in rat osteoblast cultures (Centrella *et al.* 1989) and increases bone matrix deposition in cultured calvaria (Pfeilschifter *et al.* 1990). In an *in vivo* study, PDGF was also found to enhance bone formation in

subcutaneously implanted matrix (Howes *et al.* 1988). Insulin-like growth factor-I (IGF-I) has also been proposed to be a major stimulator of bone formation (Isgaard *et al.* 1986, Schlechter *et al.* 1986, Spencer *et al.* 1991). IGF-I functions as both an autocrine and paracrine regulator to promote the growth and differentiation of bone cells (Canalis 1980, Hock *et al.* 1988). It is generally believed that PDGF is a competence factor and IGF-I is a progression factor. Both factors act via different receptors and regulate different processes of bone formation. The question arises, then, of whether the two factors may interact and collaborate to enhance the lineage of osteoblast cells and stimulate bone formation. There are several reports of a combined effect of PDGF and IGF-I. Canalis *et al.* (1989) demonstrated that the combination of the two factors had an additive effect on DNA synthesis in rat calvaria cells. A combined effect on the stimulation of bone matrix apposition *in vitro* was also reported (Pfeilschifter *et al.* 1990). Using stromal cell cultures, we were able to show that IGF-I and PDGF can stimulate cell proliferation in an additive manner (Tanaka & Liang 1995). In an *in vivo* study, the synergistic action of PDGF and IGF-I was demonstrated in wound healing (Lynch *et al.* 1987), periodontal bone formation (Lynch *et al.* 1991) and bone repair in a cortical defect model (Lynch *et al.* 1994). However, in order to assess the individual, additive, or synergistic effects of growth factors on bone formation at the cellular and molecular levels, histologic analysis as used in most previous studies is insufficient, since the effect is expressed as the net result of a number of cellular events.

In the past, we have documented the use of a marrow ablation model to investigate the age-related impairment in osteogenic capacity (Liang *et al.* 1992). This procedure induces rapid proliferation of osteoblasts and medullary bone formation and allows us to assess the process of osteogenic differentiation *in vivo* at both the cellular and molecular levels. Our previous results using this model showed that the mRNA levels of osteoblast markers were decreased in old rats and were partially restored by an IGF-I infusion (Liang *et al.* 1992, Tanaka *et al.* 1994).

The present study was undertaken to explore the possibility that growth factor can be used to restore the age-related impairment in bone formation activity. We infused PDGF and IGF-I individually and in combination into the marrow cavity after marrow ablation to determine whether the combination of PDGF and IGF-I is superior to the use of either factor alone in stimulating the expression of osteoblast markers.

Materials and Methods

Animals

Adult (6-month-old) and old (24-month-old) male Wistar rats were supplied by the Animal Facility, Gerontology

Research Center, National Institute on Aging. We used male rats to avoid the possible influence of decreased estrogen secretion in female rats, which may complicate the interpretation of the results. Throughout the experiment, the animals were housed with a cycle of 12 h of light and 12 h of darkness, and fed *ad libitum* standard National Institute of Health rat chow consisting of 23.5% protein, 1.2% calcium and 1.0% phosphate.

Bone marrow aspiration

Femoral bone marrow was aspirated as described previously (Patt & Maloney 1975). Briefly, the rats were anesthetized with ketamine (50 mg/kg) and xylazine (10 mg/kg). A longitudinal incision was made across the medial aspect of the knee to expose the bone. A 1.6 mm hole was drilled through the femoral intercondylar space into the marrow cavity. The marrow was removed through a cannula inserted through the hole and attached to a vacuum line, then the marrow cavity was irrigated with saline. For the growth factor experiment, an Alzet minipump containing growth factor solution was inserted subcutaneously on the back of the rat adjacent to the hip joint. A polyethylene catheter was used to deliver growth factor into the right femoral cavity. The left femur was used as a control. The skin wound was closed with wound clips. Human recombinant PDGF-B homodimer (Bachem, Inc., Torrance, CA, USA) and IGF-I (Bachem, Inc.) were infused for 5 days. The rats were killed at day 6 after the surgical procedure. Both femora were removed and stored at -80°C until they were processed. The minipumps were recovered and the residual solution in the pump was estimated to ensure that the infusion had proceeded as expected. This protocol was approved by the Animal Care and Use Committee at the Gerontology Research Center, National Institute on Aging.

RNA preparation

Bones were pulverized with a mortar and pestle pre-chilled in dry ice. RNA was extracted from the bones as described previously (Nemeth *et al.* 1989). Briefly, the powdered bone was homogenized in a 6 M guanidine thiocyanate (Fluka Biochemika, Buchs, Switzerland) solution containing 1.5% N-laurosarcosine (Sigma Co., St Louis, MO, USA), 67.5 mM potassium acetate and 0.1% antiform A (Sigma Co.). The debris was removed by centrifugation at 8000 *g* for 40 min, and the supernatants were loaded onto a 5.6 M cesium chloride cushion. Total RNA was separated by density gradient ultracentrifugation at 145 000 *g* for 18 h. The RNA pellet was dissolved in DEPC-treated H_2O , extracted with chloroform/isobutanol, and precipitated with 70% ethanol at -20°C . The purity of the RNA was monitored by the ratio of the absorbances of the samples at 260 nm and 280 nm.

Northern analysis

Total RNA (15 µg) was denatured, separated on a 1% agarose gel (FMC Co., Rockland, ME, USA) and transferred to Gene Screen paper (New England Nuclear, Boston, MA, USA). Inserts of cDNA were released from the plasmid by restriction enzyme digestion and separated on a 0.75% agarose gel. The appropriate cDNA fragment was extracted from slices of the agarose gel with a Prep-A-Gene kit (Bio-Rad, Richmond, CA, USA). The cDNA probes used were rat alkaline phosphatase (AP) (0.6 kb, EcoRI) (Noda *et al.* 1987), rat collagen α_1 (I) (COLI) (1.3 kb, PstI) (Genovese *et al.* 1984), rat osteocalcin (OC) (0.5 kb, EcoRI) (Weinreb *et al.* 1990) and rat osteopontin (OP) (1.3 kb, EcoRI) (Yoon *et al.* 1987). ^{32}P -Labeled probes were prepared with a multiprime labeling kit (Amersham Co., Arlington Heights, IL, USA). The blots were hybridized at 42 °C for 24 h in 50% formamide. After hybridization, the blots were washed with $2 \times \text{SSC}$ (0.15 M NaCl and 0.015 M sodium citrate) and 0.1% SDS twice at room temperature, $1 \times \text{SSC}$ and 0.1% SDS at 50 °C, and $0.5 \times \text{SSC}$ and 0.1% SDS at 60 °C. Autoradiograms were exposed at -70 °C with an intensifier screen.

Dot blot analysis

Aliquots of 0.5–5.0 µg total RNA were applied onto the Gene Screen membrane with a Minifold apparatus (Schleicher & Schuell, Waburn, MA, USA). The membrane was baked at 80 °C for 2 h to immobilize the RNA and then hybridized with various cDNA probes, as described above. A polythymidylate probe was used to determine poly(A)⁺RNA (Hollander & Fornace 1990). Briefly, the ^{32}P -labeled probes were prepared by using oligo(dT)^{12–18} as primer and poly A as template to synthesize ^{32}P -poly(dT). Hybridization and washing of the dot blots followed the procedures described. The radioactivity of each dot on the filter was measured with a Betascope 603 Blot analyzer (Betagen Corp., Waltham, MA, USA). The level of each mRNA was normalized with poly(A)⁺RNA to correct for variations in the amount of RNA applied. To test whether the changes were specific, the content of poly(A)⁺RNA was also determined. No significant difference in poly(A)⁺RNA was observed between adult and old bone or between control and treated bone relative to total RNA.

Statistical analysis

All data are expressed as means $\pm$ S.E. Statistical analyses were performed with a Student's *t*-test.

Results

Effect of age on the gene expression of osteoblast markers following marrow ablation

We have previously examined the mRNA expression patterns of osteoblast markers in the bone marrow ablation

model and showed that maximal activation was observed at day 6 (Liang *et al.* 1992). To assess the effect of age on gene expression activity, we examined the mRNA levels of COLI, AP, OP and OC in the femurs at day 6 after marrow ablation. For quantitation of the mRNA levels, we analyzed the relative mRNA content by dot blot analysis. In the uninjured femurs, the mRNA levels of osteoblast markers were very low (data not shown), but the activity was enhanced significantly by marrow ablation. The mRNA levels in the injured femurs were significantly lower in the old than in the adult rats (23%, 56%, 46% and 23% respectively) (Fig. 1). The effect of age on gene expression was also analyzed by Northern blot hybridization. Figure 2 shows a representative result using randomly selected bone samples from each age group.

Effect of PDGF on the gene expression of osteoblast markers

In order to study whether growth factor can abolish the age-related deficits in mRNA expression in old bone, PDGF was infused locally into the right femur after marrow ablation and the effect on gene expression was examined. The left femur was infused with vehicle only and served as the control, while the contralateral bone was infused with 25 or 50 ng/day growth factor for 5 days. The results are expressed as the ratio of the mRNA level of the treated leg to that of the control leg. In old rats, the expression of OP mRNA was significantly stimulated by 25 and 50 ng/day PDGF (33% and 30%), whereas the expression of COLI, AP and OC was not affected by PDGF treatment. A similar PDGF effect was observed in adult rats (Fig. 3).

Effect of IGF-I on the gene expression of osteoblast markers

In adult rats, IGF-I did not have a significant effect on gene expression. In contrast, IGF-I was moderately effective in stimulating gene expression in old bones (Fig. 3). Treatment with IGF-I at 25 ng/day significantly enhanced the mRNA levels of COLI, AP and OP (41%, 20% and 38% respectively). OC mRNA was not affected by IGF-I treatment. At 50 ng/day IGF-I, the effect was similar to that obtained with 25 ng/day IGF-I.

Effect of PDGF plus IGF-I on the gene expression of osteoblast markers

In adult bones, combined treatment of PDGF with IGF-I caused an increase in the level of OP gene expression by about 20% and no change in AP, OC and COLI genes. When PDGF and IGF-I were infused into old bones at 25 ng/day, the expression of OC mRNA was significantly enhanced (38%) (Fig. 3). The two agents also had a significant effect on the mRNA expression of COLI, AP and OP (39%, 29% and 36% respectively), although these levels of mRNA were not significantly higher than those

Figure 1 Effect of age on the mRNA levels of osteoblast markers after bone marrow ablation. Bone marrow was aspirated from the femurs of adult (6-month-old; 6M) or old (24-month-old; 24M) rats. At day 6, total RNA was prepared from the femurs. The level of each specific mRNA was quantitated by dot blot analysis and normalized with poly(A)⁺RNA. Values shown are means $\pm$ S.E. for six to seven rats. * $P < 0.0001$ (unpaired *t*-test).

achieved with either IGF-I or PDGF alone. At 50 ng/day of the PDGF/IGF-I treatment, no dose-response effects were observed. The effect of the two agents on gene expression was analyzed by Northern blot hybridization, and Fig. 4 shows a representative result.

Discussion

Impairment of bone formation activity in senescence is in part due to the low level of growth factors. Although no data on the levels of PDGF are available, there is some evidence that serum levels and the expression of IGF-I decline in senescence (Rudman *et al.* 1981, Florini *et al.* 1985, Tanaka *et al.* 1996). In addition, it has been demonstrated that the age-dependent decline in the growth hormone/IGF-I/IGF-binding protein axis is associated with the age-related loss of bone mass (Sugimoto *et al.* 1997). A recent study has shown that the age-related decline in IGF-I can be attributed in part to impaired calcium absorption with age (Fatayerji *et al.*

2000). Decreased calcium absorption would eventually cause a decrease in serum calcium, resulting in a compensatory increase in parathyroid hormone in aging.

The aim of the present study was to explore the possibility that growth factor can be used to up-regulate the impaired bone formation activity in old rats. We tested the effect of growth factors on the gene expression of bone matrix proteins in femurs during the process of bone formation after marrow ablation. We have previously shown that gene expression correlated with histomorphometric evidence of bone formation in a bone marrow ablation model (Liang *et al.* 1992). Several reports have also described the correlation between the mRNA levels of characteristic osteoblast products and bone formation as assessed by bone histomorphometry (Turner & Spelsberg 1991, Wakley *et al.* 1992, Rantakokko *et al.* 1999). After the removal of the marrow, the medullary cavity is filled with a blood clot, and the osteoprogenitor cells in the region adjacent to the endosteal surface proliferate and differentiate into preosteoblasts. These cells deposit trabecular bone in the cavity, with their maximal activity

Figure 2 Representative Northern blots of femoral mRNA after bone marrow ablation. Bone marrow was aspirated from the femurs of adult (6 M) or old (24 M) rats. Total RNA was prepared from the femurs, and the level of each specific mRNA was analyzed by Northern blot hybridization. The results from three representative samples are shown. Ethidium bromide staining of ribosomal RNA (28S and 18S) in the same gel is also shown.

occurring on day 6 (Patt & Maloney 1975, Liang *et al.* 1992). Using this model, we have shown that these events are attenuated in old rats (Liang *et al.* 1992). The present study confirmed the observation that the mRNA levels of osteoblast markers were consistently lower in bones from old rats. We then tested the effect of PDGF, IGF-I and a combination of the two on the expression of osteoblast markers in order to explore the possibility that growth factors can stimulate the expression of osteoblast-related genes and, thus, can restore bone formation activity in old rats.

Our results demonstrated that the administration of PDGF directly into the femur after marrow ablation failed to enhance the mRNA levels of osteoblast markers, with the exception of OP. Our previous studies in bone marrow stromal cells have shown that PDGF stimulated OP mRNA expression in a dose-dependent manner (Tanaka & Liang 1995). Thus, the results from our *in vivo* experiments are consistent with our previous results that PDGF stimulates OP expression. In addition, PDGF also has a modest inhibitory effect on the differentiated function of osteoblasts, as previously suggested in an *in vitro* study (Canalis *et al.* 1989, Hock & Canalis 1994, Tanaka Liang 1995). That finding supports the negative results for COLI, OC and AP expression that were observed in the

present experiment. As we have shown previously, IGF-I was partially effective in stimulating gene expression in old rats (Tanaka *et al.* 1994). The infusion of IGF-I enhanced the mRNA expression of COLI, AP and OP but not OC. Thus, our data clearly show that IGF-I is more effective than PDGF in stimulating the expression of osteoblast-related genes in old bone.

The combination of PDGF and IGF-I appeared to have minimal additional effects on the specific expression of bone products as compared with IGF-I. In contrast to the IGF-I treatment, however, the expression of OC was also stimulated by the PDGF plus IGF-I combination in old bone, in addition to the expression of COLI, AP and OP. Neither IGF-I nor PDGF alone was able to stimulate OC expression. Since OC is known to be produced by mature osteoblasts and to regulate the calcification of bone tissue, it is possible that the PDGF plus IGF-I treatment may stimulate maturation and mineralization in the process of bone formation. Our assumption is supported by a previous report that found that the PDGF plus IGF-I combination enhanced the density of mineralization in the callus, which was not affected by either PDGF or IGF-I alone (Lynch *et al.* 1994). In addition, it should be noted here that, since high doses of growth factors have undesirable side-effects, it would be beneficial if the

Figure 3 Effect of IGF-I and PDGF administered *in vivo* on the expression of osteoblast markers. Bone marrow was aspirated from the femurs of adult (6M) or old (24M) rats. One leg served as the control and the contralateral leg was infused with 25 or 50 ng/day of the growth factors for 5 days. The level of each specific mRNA was quantitated by dot blot analysis and normalized with poly(A)⁺RNA. Results are expressed as the ratio of the treated leg mRNA to the control leg mRNA. P=PDGF; I=IGF-I. Values shown are the means $\pm$ S.E. for six to seven animals. * $P < 0.05$ vs control (paired *t*-test).

combination of growth factors could result in a reduced dose of each growth factor.

In our present study, the treatment of growth factors was not effective in stimulating the mRNA expression of osteoblast-related genes other than osteopontin in adult rats. These age-dependent effects may be explained by the fact that the level of the growth factors may be altered in old rats, as mentioned earlier in this discussion. Moreover, using the marrow ablation model, we also observed that IGF-I gene expression in the process of bone formation decreased in old rats (Tanaka *et al.* 1996). In this regard, it is possible that the expression of osteoblast-related genes may be fully activated, probably due to the relatively high level of the serum proteins of growth factors after bone marrow ablation in adult rats, and that an additional infusion of growth factors may not be able to induce further enhancement. Indeed, the mRNA levels of osteoblast markers were consistently higher in bones from adult rats, as shown in the present study. A previous study demonstrated a similar age-related difference in the

responses to growth factors (Spencer *et al.* 1991). In that study, IGF-I infusion stimulated both cortical bone formation and trabecular bone apposition in 12-month-old rats, but not in 6- to 7-week-old rats.

Treatment with the PDGF plus IGF-I combination in old rats was partially effective in restoring the age-related decrease in the gene expression of osteoblast markers, but the actual mRNA level of treated limbs in old rats was still lower than that in untreated adult rats. Given the complexity of bone formation and the many growth factors involved in the regulation, it is reasonable that other growth factors may also be involved in reduced bone formation activity in senescence. Furthermore, the effect of growth factors may be influenced by the timing or duration of their administration in the experiment. Although we analyzed gene expression at day 6 because the maximal activation of gene expression was observed in our previous experiment at this time, it is possible that a shorter or longer period of treatment or sequential treatment may produce a more pronounced cellular response.

Figure 4 Northern blot analysis of femoral mRNA after treatment with PDGF plus IGF-I in old rats. The bone marrow was aspirated from femurs. One leg served as the control and the contralateral leg was infused with 25 ng/day PDG plus IGF-I for 5 days. Total RNA was prepared from the femurs and the level of each specific mRNA was analyzed by Northern blot hybridization. The results from two representative samples are shown. Ethidium bromide staining of ribosomal RNA (28S and 18S) in the same gel is shown at the bottom. (–) control; (+) PDGF plus IGF-I.

Thus, the stimulation of gene expression by treatment with the PDGF plus IGF-I combination in the present study should be viewed as a limited result rather than as indicative of the results that might be achieved with more refined interventions.

In conclusion, the results from our *in vivo* model clearly show that IGF-I is a better stimulator of osteoblast marker expression than is PDGF in old bone. Treatment with the PDGF plus IGF-I combination partially restored the deficit in gene expression activity in old bone, but the effect was only slightly better than that of IGF-I alone. Additional investigations in combination with other growth factors will be needed to establish the efficacy and utility of growth factor treatment for age-related bone loss.

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