Trimer Carboxyl Propeptide of Collagen I Produced by Mature Osteoblasts Is Chemotactic for Endothelial Cells*

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During the second phase of osteogenesis in vitro, rat osteoblasts secrete inducer(s) of chemotaxis and chemoinvasion of endothelial and tumor cells. We report here the characterization and purification from mature osteoblast conditioned medium of the agent chemotactic for endothelial cells. The chemoactive conditioned medium specifically induces directional migration of endothelial cells, not affecting the expression and activation of gelatinases, cell proliferation, and scattering. Directional migration induced in endothelial cells by conditioned medium from osteoblasts is inhibited by pertussis toxin, by blocking antibodies to integrins α1, β1, and β3, and by antibodies to metalloproteinase 2 and 9. The biologically active purified protein has two sequences, coincident with the amino-terminal amino acids, respectively, of the α1 and of the α2 carboxyl propeptides of type I collagen, as physiologically produced by procollagen C proteinase. Antibodies to type I collagen and to the carboxyl terminus of α1 or α2 chains inhibit chemotaxis. The chemoattractant is the propeptide trimer carboxyl-terminal to type I collagen, and its activity is lost upon reduction. These data illustrate a previously unknown function for the carboxyl-terminal trimer, possibly relevant in promoting endothelial cell migration and vascularization of tissues producing collagen type I.

Vascularization and angiogenesis implicate directional migration of endothelial cells, their proliferation, and morphogenesis of the vessels (1). A number of factors have been shown to be capable of promoting each of these events, and some factors have been shown capable of inducing more than one of them (1–3). Factors involved in regulating angiogenesis act in autocrine and paracrine fashion. The production of chemotractions by organs and tissues to be vascularized, coupled with the production of morphogenetic and mitogenic factors by these organs and/or the endothelial cells, is the emerging rule for control of vascularization in physiological and pathological circumstances (1–3). During the formation of long bone, there is transformation of the perichondrium around the cartilage model to a periosteum with bone being laid down at the mid-shaft region. At the outer surface of the cartilage model, near its center, blood vessels invade the calcified cartilage that is eroded, opening up a marrow cavity. Epiphyseal growth cartilages are then established to promote endochondral ossification, and angiogenesis occurs at the growth plate. Vascularization in endochondral ossification of the growth plates has been shown to depend on the function of VEGF1 (1) and the expression of metalloproteinase-9. Vascularization at the diaphyseal region and formation of bone marrow were not reported to be affected in mice null for these genes (4, 5).

In vitro early passage tibia-derived rat osteoblasts secrete in a developmentally regulated fashion, during the second phase of osteogenesis and coinciding with the highest level of synthesis of type I collagen, substance(s) promoting chemotaxis and chemoinvasion of endothelial, melanoma, breast, and prostatic carcinoma cells (6–9). We here report that products secreted by rat osteoblasts activate directional migration, specifically, in endothelial cells via a Gαζ/Gζ protein-dependent pathway. Chemotaxis requires the function of metalloproteinases 2 and 9 and of integrins α1, β1, and β3 and is inhibited in presence of the corresponding specific antibodies.

We have purified the chemotactic agent for endothelial cells produced by rat osteoblasts as a 120-kDa protein and obtained from it two sequences that coincide with those of the α1 and α2 C-terminal chains of procollagen type I, starting from the NH2 terminus produced by the action of procollagen C-proteinase (also bone morphogenetic protein-1; Refs. 10 and 11). The chemotactic activity is associated with the trimeric form of the carboxyl-terminal peptide of collagen type I (C3; Ref. 10) and is inhibited by antibodies to collagen type I and to either of its carboxyl-terminal chains. These results identify a novel and unknown function for the processed carboxyl fragment of type I collagen and underline again the multifunctional role of collagen molecules, as already shown for fragments physiologically produced from collagen type XVIII (endostatin; Ref. 12), collagen type II (chondrocalcin; Refs. 13 and 14), collagen type XV (restin; Ref. 15), and collagen type IV (canstatin; Ref. 16).

EXPERIMENTAL PROCEDURES

Cells and Culture Conditions—Rat tibial osteoblasts cultures (ROB) and the characterization of their osteogenic phenotypes were described previously (6, 7). Differentiation medium was Coon’s modified F-12 supplemented with 10% fetal calf serum (Seromed, Italy), ascorbic acid (100 μg/ml), and β-glycerophosphate (10 mM). Osteoblasts were metabolically labeled for 5 h in methionine and cysteine and serum-free medium with Trans35S-labeled amino acids (ICN) at 200 μCi/ml.

EA hy926 endothelial cells (from Dr. M. Soria, Milan, Italy), primary keratinocytes (from Dr. M. De Luca, Roma, Italy) and Madin-Darby canine kidney cells (from Dr. G. Gaudino, Novara, Italy) were cultured in Dulbecco’s modified Eagle’s medium, 10% fetal calf serum. Human umbilical vein endothelial cells (from Dr. De Filippis, Torino, Italy)
were cultured on a coating of 0.1% gelatin from bovine skin, in M199 medium supplemented with 20% fetal calf serum, 50 μg/ml of endothelial cell growth supplement, and 100 μg/ml heparin. IG11 (from Dr. A. Vecchi, Milano, Italy) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 20% fetal calf serum, 2 mM glutamine, 2 mM sodium pyruvate, 1% of essential amino acids, 20 mM HEPES, 50 μg/ml endothelial cell growth supplement, and 100 μg/ml heparin.

**Conditioned Media from Osteoblasts—**Conditioned media (CM) were collected from mature osteoblasts in the second phase of osteogenesis (7–10 days) in vitro from ROB propagated in vitro. After rinsing the monolayer two times with warm phosphate-buffered saline, the cultures were incubated in serum-free medium (SFM), and the CM was harvested, centrifuged to remove cells and debris, and immediately utilized or kept frozen. When indicated, tunicamycin (5 μg/ml) was added to the culture at 6 days of differentiation and maintained for 20 h in medium containing serum and for the following 5 h in SFM during collection of CM.

**Chemotaxis Assay—**We have measured the induction of chemotaxis in a Boyden chamber assay (17), measuring the migration of target cells through gelatin (5 ng/ml-coated polycarbonate polivinylpyrrolidone-free filters (12-mm pore size; Nucleapore, Italy). Standard conditions for Boyden chamber assays utilized human endothelial line EA hy926, 12 × 10^4 cells/upper compartment and 17 μg of DNA equivalent of CM/upper compartment of each chamber. Incubation was for 6 h. The upper chamber filter was scrapped, and the migrated cells were fixed in ethanol, stained with toluidine blue (2%), and quantitated microscopically at a ×20 magnification. Five random fields were counted. 100 cells/field corresponded to 15% of the total cells. When indicated, other cell lines were tested in the same conditions. Each experiment was in duplicate and was repeated at least three times.

**Characterization of the Chemotactic Activity and Treatments of CM—**The effect of pH variation on the chemotactic activity of CM was tested at room temperature under stirring for 3 min by the addition of NaOH or HCl in predetermined amounts to obtain the desired pH, followed by neutralization before use in a biological assay. The effect of EDTA or β-mercaptoethanol was tested by adding it to CM that was then directly utilized in a chemotactic assay. Heat lability was tested by setting 1 ml of CM in a preheated bath for 15 min under stirring, followed by chilling in ice.

**Trypsin digestion was for 30 min at 37 °C, followed by soy bean inhibitor (4.1 mM). Chondroitinase (1 unit/ml) digestion was for 60 min at 37 °C. Heparin (10 μg/ml or 50 μg/ml) was added directly to CM.**

Antisera against human fibronectin (from Prof. G. Tarone, Torino, Italy) or rat type I collagen (Pasteur, Lyon, France) or α1 and α2 carboxy-terminal propeptides of collagen type I (from Dr. A. Veis, Chicago) or goat IgG (Pasteur) were added to CM before utilizing in a Boyden assay. All of the concentrations of the antisera utilized to test their effects on endothelial cell migration in Boyden assays were also tested in SFM and showed no effects on cell migration or viability.

**Scattering Test—**The scattering effect of osteoblast CM at various dilutions was tested on Madin-Darby canine kidney cells, human keratinocytes, and EA hy926 colonies over 18 h and estimated by microscopy. As a positive control, hepatocyte growth factor (HGF; from Dr. P. Campoglio, Torino, Italy) was used.

**Endothelial Cell Treatments—**During Boyden chamber assay in standard conditions, EA hy926 were added with pertussis toxin, antiserum against metalloproteinase 2 or 9 (from Dr. Stettler-Stevenson), or blocking antibodies against integrins α1 (anti-rat 3A5; from Dr. C. Damsky), α2 (anti-human; Chemicon), β1 (anti-human BVT and AIB2; from Dr. Tarone), and β3 (anti-human, from Dr. Tarone) at the dilutions indicated in the figures.

**Gelatin Zymography of CM—**Endothelial cell CM was collected after incubation for 6 h from the upper compartment of Boyden chambers. The lower compartments contained, respectively, CM SFM, from osteoblasts, or purified C3. EA hy926 conditioned media were electrophoresed on 10% SDS-acrylamide gels containing 2.8 mg/ml gelatin in a water-refrigerated box. After electrophoresis, the gels were washed twice for 30 min each time in 2.5% Triton X-100, incubated overnight at 37 °C in 50 mM Tris, pH 7.5, 0.2 mM NaCl, 10 mM CaCl2, 1 mM ZnCl2, 0.02% NaN3. Proteolytic activity was visualized as clear bands against the blue background after staining the gels in 0.2% Coomassie Blue R-250 in 40% methanol and 10% acetic acid, followed by destaining in 40% methanol and 10% acetic acid.

**Fractionation of CM and Purification of C3—**CM were collected for 5 h from propagated ROB (46–70 passages in vitro) in SFM differentiation medium. Molecular weight fractionation of CM was performed by centrifugation in Centrifil (Millipore Corp.) tubes, fitted with molecular sieve filters, according to the instructions of the manufacturer.

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**FIG. 1.** The activity of osteoblasts CM on endothelial cells is developmentally regulated, is mostly chemotactic, and has endothelial cells as specific targets. A, time course of the expression of chemotactant during in vitro osteogenesis by Boyden chamber assay. B, checkerboard test by standard Boyden assay. Vertical arrows identify chemotactic attraction, the diagonal line indicates chemokinetics. C, standard Boyden assay with CM (white bars) and SFM (black bars) utilizing different cell types. Error bars indicate S.E.

**RESULTS**

During the osteogenic differentiation, in vitro propagated ROB populations express transiently a chemotactic activity for endothelial cells (Fig. 1A). By the checkerboard test we show that the major component of the activity is chemotactic, with only a minor chemokinetic component (Fig. 1B). Biological tests do not identify scattering (Table I) and mitogenic (not shown) activities in chemotactic CM at the concentration that is inducive of migration. Presence or absence of serum is irrelevant to the effect of CM on proliferation of EA hy926, human umbilical vein endothelial cells, and 1G11 cells of murine lung endothelium. The chemotactic response is specific for endothelial cells, among the normal cells tested (Fig. 1C).

The response of endothelial cells to the chemotactrant is inhibited in a dose-dependent fashion by pertussis toxin (PTX) (Fig. 2A) in blocking monoclonal antibodies to α1, β1 integrins and by a polysaccharide antiserum against β3 integrin (Fig. 2B). Antibodies against MMP-2 and MMP-9 also inhibit migration in chemotaxis assay (Fig. 2C).

Analysis of the chemico-physical parameters of the chemotactrant shows loss of activity upon short treatment at acid pH and upon reduction (Table II). Chemotaxis induced by CM is
The addition of SFM containing HGF or CM to cell colonies was followed by incubation at 37 °C. The colonies were inspected for scattering at 3, 6, and 18 h after the medium change, and the results listed are for the effect at 18 h. MDCK, Madin-Darby canine kidney cells, b) EA hy926, and c) human keratinocytes. +, scattering; −, lack of scattering; ND, not done.

### Table I

| Treatment | Inhibition |
|-----------|------------|
| 50 °C     | 21         |
| 65 °C     | 84         |
| 80 °C     | 91         |
| pH 3      | 30         |
| pH 5      | 35         |
| pH 8      | 0          |
| pH 10     | 7          |
| β-Mercaptoethanol (1 mM) | 21 |
| β-Mercaptoethanol (5 mM) | 38 |
| β-Mercaptoethanol (10 mM) | 44 |
| β-Mercaptoethanol (20 mM) | 59 |
| EDTA (20 mM) | 0.2 |
| EDTA (100 mM) | 0  |

**Fig. 2.** Requirement for chemoinduction of G, G, protein activity, integrins, and MMP-2 and -9 function. A standard Boyden assay with CM and EA hy926 is shown. A, cells were treated with the indicated concentrations of pertussis toxin (PTX) during assay (6 h). B, chemotaxis was assayed in the presence of the indicated integrin subunit-blocking antibodies, added to the upper compartment of the Boyden chamber (1× to 5× indicate the relative concentrations of the antibody). C, chemotaxis was assayed in the presence of antisera against MMP-2 or MMP-9 added to the upper compartment of the Boyden chamber. In A–C, untreated corresponds to lack of any treatment, and C represents the noninducing SFM, to account for random migration. Error bars indicate S.E.

unaffected by the addition of heparin (Fig. 3A), and the activity is independent from glycosylation of osteoblast proteoglycans (Fig. 3, B and C).

Fractionation on molecular sieve enriches the activity in a fraction of size above 100 kDa (Table III, left). The activity binds to heparin-Sepharose and is eluted at the concentration of 0.5 mM NaCl in a stepwise gradient (Table III, right). All of the above chemico-physical and biological characteristics are distinguished from those of all known chemoattractants for endothelial cells (VEGF/VP, fibroblast growth factors, insulin-like growth factors, platelet-derived endothelial growth factor, transforming growth factor-β, tumor necrosis factor-α, angiotropin, HGF, granulocyte-macrophage colony-stimulating factor, and angiopoietin-1).

Purification of the chemoactive substance from the osteoblast CM has been performed by molecular sieve fractionation, followed by heparin-Sepharose chromatography with elution with a linear gradient of NaCl. The fractions have been tested for inducing chemotaxis of EA hy926 cells in the standard assay in a Boyden chamber; active fractions eluted between 0.42 and 0.58 mM NaCl. The average yield of protein after the purification was 1.5% of the starting amount. Fig. 4A illustrates the purification by heparin-Sepharose chromatography.
of a preparation of CM metabolically labeled with Tran$^{35}$S-labeled amino acids, and Fig. 4B shows that the single band at about 120 kDa obtained by gel electrophoresis from the unreduced sample splits into two bands at 33 and 35 kDa upon reduction.

In large scale preparations, after purification the reduced preparative gels show a single large band spanning 33–35 kDa. This has been sequenced twice and yielded consistently a double amino-terminal sequence; amino acid residues have been attributed to each sequence, taking advantage of the different amount. The major sequence has provided clearly identifiable residues up to position 20, whereas the minor sequence has been identified up to residue 12. The first sequence corresponds in the EMBL data bank to rat type I procollagen α₁ chain, starting at position 1208, and the second sequence corresponds to rat type I procollagen α₂ chain, starting at position 1126 (Fig. 4C). The amino termini of both sequences overlap with those produced in the processing of type I procollagen in physiological conditions and in vitro by procollagen C-proteinase (bone morphogenetic protein-1). The α₁ and α₂ chains contain, respectively, 246 and 247 amino acid residues, compatible with the molecular weights observed when the purified fraction is run on reduced SDS-polyacrylamide gel electrophoresis. The molecular size of the active purified protein is compatible with it being a trimer of two α₁ and one α₂ carboxy-terminal portions of collagen type I. The loss of the chemotactic activity observed in CM by treatment with reducing agents shows that the conservation of intramolecular S–S bonds is required for preservation of the activity. Hence, the biologically active molecule is the trimeric form of the carboxy propeptide of type I collagen (C3). In agreement with the identification of the chemotactrant from CM as C3, the chemotaxis of endothelial cells is inhibited in a dose-dependent fashion by the addition to CM of antibodies against collagen type I and by antibodies directed to α₁ and α₂ carboxy-terminal propeptides of type I collagen, while no significant inhibition is determined by antibodies against fibronectin and by an aspecific IgG (Fig. 5). No changes of the pattern of cell-associated and secreted metalloproteinases expressed by EA hy926 nor of their activation state are detected upon exposure to CM or C3 (Fig. 6). MMP-2 and MMP-9 are secreted by the endothelial cells, and their activated derivatives at 69, 62, and 59 kDa, respectively, for MMP-2 and 82 kDa for MMP-9 are detected. Also, expression of MMP-14, the membrane metalloproteinase at 66 kDa, is unchanged, regardless of chemoinduction, in the cell lysates analyzed by zymography on gelatin.

### Table III

| Molecular size | Cells/field | NaCl | Cells/field |
|----------------|-------------|------|-------------|
| <30 kDa        | 10 ± 5      | 0.1  | 0           |
| 30–50 kDa      | 42 ± 1      | 0.5  | 91 ± 4      |
| 50–100 kDa     | 38 ± 1      | 1.0  | 12 ± 3      |
| >100 kDa       | 109 ± 4     | 1.5  | 16 ± 2      |
| Unfractionated | 104 ± 5     | 2.0  | 17 ± 2      |
| SFM            | 30 ± 3      |      |             |

### DISCUSSION

We show that the purified carboxy-terminal propeptide trimer of collagen type I plays a previously unsuspected biological role as chemotactrant toward endothelial cells. During osteogenesis in vitro, C3 molecules are generated as a by-product of collagen biosynthesis by mature osteogenic cells, and chemotactrant activity in CM is detectable in concomitance with maximum expression and deposition of collagenous matrix by mature osteoblasts and decrease at mineralization of the cultures (6, 7). We have described a similar developmentally associated pattern of expression of a chemotactrant for endothelial cells and tumor cells produced by ROB in primary cultures (8, 9). We also detected a similar pattern of chemotactrant expression by analysis of the chemotaxis of endothelial cells induced by CM collected during in vitro osteogenesis of
is up-regulated by VEGF in endothelial cells (28). We demonstrate an inhibitory effect on migration of endothelial cells using antibodies to $\alpha_1$ and $\beta_1$ integrins, not present with antibodies against $\alpha_2$ integrin. Since we have utilized only one blocking monoclonal antibody against $\alpha_2$ integrin, this last finding is not conclusive about the relevance of $\alpha_2$ integrin for endothelial cell directional migration.

The inhibitory effect of blocking antibodies to $\alpha_1\beta_1$ integrin on the migration of endothelial cells induced by osteoblast CM suggests that $\alpha_1\beta_1$ integrin could be involved in the recognition of the chemoattractant by endothelial cells. Nonetheless, endothelial cells might require $\alpha_2\beta_1$ integrin to cross into the gelatin coating the filters, and further testing is required to discriminate between these possibilities.

The expression of $\beta_3$ integrin is required for the osteoblast CM-induced migration of endothelial cells. Morphogenesis of vessels in vitro, as well as VEGF-induced and VEGFR2-mediated activation of phosphatidylinositol 3-kinase and proliferation of endothelial cells, require the expression of $\beta_3$ integrin (27, 28). We do not presently know what role $\beta_3$ integrin might play in the induction of migratory response by C3.

Metalloproteinases are also required for endothelial cell migration induced by osteoblast CM. Expression of MMP-14, the membrane metalloproteinase-1, was shown to be required for endothelial cell invasion of fibrin gels in vivo and in vitro independently from MMP-2 expression (29). In EA hy926, the expression of MMP-2, MMP-9, and of the membrane gelatinase MMP-14 are constitutive and are not changed by exposure of the cells to chemoattractant CM from osteoblasts and to purified C3. Also, the activation of MMP-2 and MMP-9 occur constitutively, suggesting that the proteases required for EA hy926 migration are not under the control of the chemotactic stimulus. Their inhibition will anyhow result in hindrance to migration through the gelatin on the filter. Unlike in the case of EA hy926, osteoblast CM induces MMP-9 expression in melanoma, breast, and prostatic carcinoma cells; in these last cases, induction of urokinase-type plasminogen activator was also detected (8, 9). C3 is not mitogenic in vitro at the concentrations inductive of chemotaxis, and we are presently testing concentrated CM for mitogenic activity. Nonetheless, synergism of function of different angiogenic stimuli was reported (1–3); bone cells produce and secrete in their extracellular matrix other factors that have been implicated in the control of vascularization, such as osteopontin (30), angiopoietin-1 (31), and transforming growth factor-$\beta_1$ (32) and other growth factors that may participate or influence this process and stimulate endothelial cell proliferation. Endothelial cells might therefore find in vivo in the bone extracellular matrix the stimuli required for proliferation and morphogenesis.

In summary, previous evidence pointed primarily to a role of C3 as feedback regulator of type I procollagen synthesis in homeostasis during bone development and metabolism (33–35) and in mineralization (36), and our results show that C3 has an additional role in promoting specifically directional migration of endothelial cells.

Production of C3 is specifically associated with the mature osteoblastic phenotype, and the chemotactic activity of C3 might have a role in promoting in vivo localized vascular invasion in the areas of newly formed bone during embryogenesis. Since the factors required for the angiogenic invasion of the growth plate are not required for the initial vascularization at the diaphyseal bone collar and for the formation of the bone marrow cavity (4, 5), it is likely that other mechanisms are involved in determining the vascular invasion in the collagen type I-based extracellular matrix deposited by osteoblasts and periosteal cells at this site. Production of C3 might represent

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**Collagen I Pro-C Trimer Is Endothelial Cells Chemoattractant**

The chemotactic response of endothelial cells requires the activation of GzGz-proteins, the functionality of integrins $\alpha_1$, $\beta_1$, and $\beta_3$, and the functionality of metalloproteinases 2 and 9. G protein signaling events activated by chemoattractants have been described, and it was suggested that G-proteins can generally act as mediators of the cellular response to chemotactic stimuli (18). G-proteins are known to synergize in the cellular response to growth factors, cytokines, and integrins, through a mechanism as yet unknown. Synergy with other receptors might be involved also in the chemotactic response to C3, and $\beta_3$ integrin receptors are likely candidates.

$\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins were shown to interact with collagen carboxyl-terminal propeptides (19, 20). $\alpha_1\beta_1$ acts through Shc, a mitogen-activated protein kinase (21). $\alpha_2\beta_1$ acts by a phosphotyrosine-dependent pathway in regulating type I procollagen synthesis and in inducing collagenase (22–24) and is downregulated in presence of high calcium levels (25). Synthesis of both integrins

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2 D. Palmieri, V. Ulivi, and P. Manduca, unpublished observation.
the initial chemotactic event to orient endothelial cells toward the bone collar in the long bone. The role of osteoblasts and periosteal cells in promoting vascular invasion in vivo requires investigation.

The finding that a metabolically produced fragment of collagen type I may have a specific biological role is not so unexpected in view of the fact that specific roles were already reported for fragments physiologically produced from various collagens (12–16). For example, endostatin from collagen type XVIII, restin from collagen type XV, and canstatin from collagen type IV are involved in the control of vascularization. In general, the multiple functions of collagen molecules might be therefore the outcome of a selection during evolution, which allows structural multidomain proteins to maintain homeostasis of the vascularization (and growth?) of tissues.

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