THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 42, Issue of October 20, pp. 32658–32663, 2000
Printed in U.S.A.
© 2000 by The American Society for Biochemistry and Molecular Biology, Inc.

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

Received for publication, March 29, 2000, and in revised form, July 25, 2000
Published, JBC Papers in Press, August 2, 2000, DOI 10.1074/jbc.M002698200

Daniela Palmieri‡, Laura Camardella§, Valentina Ulivi‡, Gaetana Guasco‡, and Paola Manduca‡¶

From ‡Genetica, Dipartimento di Oncologia, Biologia e Genetica, Università di Genova, C. Europa 26, 16132, Genova and §IBPE, Consiglio Nazionale delle Ricerche, via Marconi 10, 80100 Napoli, Italia

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 α_1, β_1, and β_3, 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 α_1 and of the α_2 carboxyl propeptides of type I collagen, as physiologically produced by procollagen C proteinase. Antibodies to type I collagen and to the carboxyl terminus of α_1 or α_2 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 chemoattractants 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 midshaft 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 VEGF[1] (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 G_o/G_i protein-dependent pathway. Chemotaxis requires the function of metalloproteinases 2 and 9 and of integrins α_1, β_1, and β_3 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 α_1 and α_2 C-terminal chains of procollagen type I, starting from the NH_2 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 procollagen 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-glycerolphosphate (10 mM). Osteoblasts were metabolically labeled for 5 h in methionine and cysteine and serum-free medium with Tran^{35}S-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 Filipppi, Torino, Italy)

* This work was supported by the Progetto Nazionale MURST and Progetto Biotecnologie, Consiglio Nazionale delle Ricerche. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence and request for materials should be addressed: Prof. Genetica, Dip. Oncologia, Biologia e Genetica, Università di Genova, 26 C. Europa, Palazzo delle Scienze, 4° piano, 16132 Genova, Italy. Tel./Fax: 39-010-3538240; E-mail: man-via@igecuniv.csita.unige.it.

[1] The abbreviations used are: VEGF, vascular endothelial growth factor; ROB, rat osteoblast(s); HGF, hepatocyte growth factor; C3, carboxyl propeptide of collagen type I; CM, conditioned medium/media; SFM, serum-free medium; MMP, matrix metalloproteinase.

This paper is available on line at http://www.jbc.org

Downloaded from http://www.jbc.org/ by guest on July 25, 2018

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. 1G11 (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, 100 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 for 5 h in serum-free medium (SFM), and the CM was harvested, centrifuged to remove cells and debris, and immediately utilized or kept frozen. When indicated, tunicamicin (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 polyvinylpyrrolidone-free filters (12-nm pore size; Nucleopore, 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/lower compartment of each chamber. Incubation was for 6 h. The upper side of filters was scraped, 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 (4.1 mM) 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 α_1 and α_2 carboxyl-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. Comoglio, Torino, Italy) was used.

Endothelial Cell Treatments—During Boyden chamber assay in standard conditions, EA hy926 were added with pertussis toxin, antisera against metalloproteinase 2 or 9 (from Dr. Stettler-Stevenson), or blocking antibodies against integrins α_1 (anti-rat 3A3; from Dr. C. Damsky), α_2 (anti-human; Chemicon), β_1 (anti-human BV7 and AIIB2; from Dr. Tarone), and β_3 (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 M NaCl, 10 mM $CaCl_2$, 1 mM $ZnCl_2$, 0.02% NaN_3. 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 Centriplus (Millipore Corp.) tubes, fitted with molecular sieve filters, according to the instructions of the manufacturer.

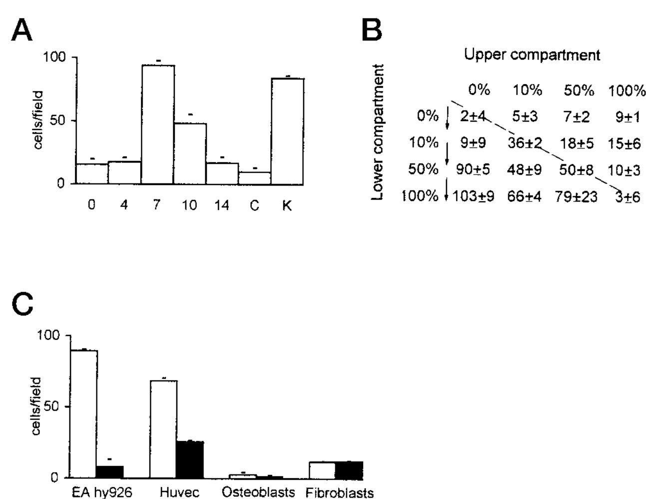

FIG. 1. **The activity of osteoblasts CM on endothelial cells is developmentally regulated, is mostly chemotactic, and has endothelial cells as specific targets.** *A*, time course of the expression of chemoattractant during *in vitro* osteogenesis by Boyden chamber assay. *0–14* refer to days of osteoblast differentiation when serum-free CM were collected; *C*, fresh serum-free medium, negative control; *K*, CM from Kaposi cells, positive control. *B*, checkerboard test by standard Boyden assay. *Vertical arrows* identify chemoattraction, the *diagonal line* identifies chemokinesis. *C*, standard Boyden assay with CM (*white bars*) and SFM (*black bars*) utilizing different cell types. *Error bars* indicate S.E.

1–10 ml of CM or fractions recovered from Centriplus were loaded, after extensive dialysis at 4 °C against 50 mM Tris-HCl, on a 2.5-ml column of heparin-Sepharose and eluted stepwise or with a linear gradient of NaCl (0–1 M) in Tris-HCl 50 mM, pH 7.5, at 4 °C. The fractions were dialyzed against SFM at 4 °C 16 h before testing for chemotactic activity or concentrated by ethanol precipitation before gel electrophoresis on SDS-polyacrylamide gels.

Purification for sequencing purposes was done from 500 ml of CM, and the above procedure was scaled up accordingly. Preparative gels were electrotransferred on ProBlott™ membrane (Applied Biosystems), and proteins were revealed by Coomassie staining. Excised bands were directly sequenced by automated Edman degradation on a Procise Protein Sequencer, model 492, equipped with a 140C Microgradient system from PerkinElmer Life Sciences. The EMBL data bank was utilized for sequence assignment. Type I collagen α_1 chain has accession number Z78279.1, and the α_2 chain has accession number AF121217.1. All data were the average of at least duplicate samples. Experiments were repeated at least twice. All chemicals were from Sigma unless otherwise specified.

RESULTS

During the osteogenic differentiation, *in vitro* propagated ROB populations express transiently a chemotactic activity for endothelial cells (Fig. 1*A*). By the checkerboard test we show that the major component of the activity is chemotactic, with only a minor chemokinetic component (Fig. 1*B*). Biological tests do not identify scattering (Table I) and mitogenic (not shown) activities in chemotactic CM at the concentration that is inductive 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. 1*C*).

The response of endothelial cells to the chemoattractant is inhibited in a dose-dependent fashion by pertussis toxin (*PTX*) (Fig. 2*A*) by blocking monoclonal antibodies to α_1, β_1 integrins and by a polyclonal antiserum against β_3 integrin (Fig. 2*B*). Antibodies against MMP-2 and MMP-9 also inhibit migration in chemotaxis assay (Fig. 2*C*)

Analysis of the chemico-physical parameters of the chemoattractant shows loss of activity upon short treatment at acid pH and upon reduction (Table II). Chemotaxis induced by CM is

Downloaded from http://www.jbc.org/ by guest on July 25, 2018

TABLE I
Scattering test

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. MDKC, Madin-Darby canine kidney cells, b) EA hy926, and c) human keratinocytes. +, scattering; −, lack of scattering; ND, not done.

	MDCK	EA hy926	Human keratinocytes
HGF (0.8 units/ml)	+	−	ND
HGF (0.16 units/ml)	+	−	ND
HGF (0.08 units/ml)	−	−	ND
HGF (0.016 units/ml)	−	−	ND
CM undiluted	−	−	−
CM (75%)	−	−	−
CM (50%)	−	−	ND
CM (25%)	−	−	ND

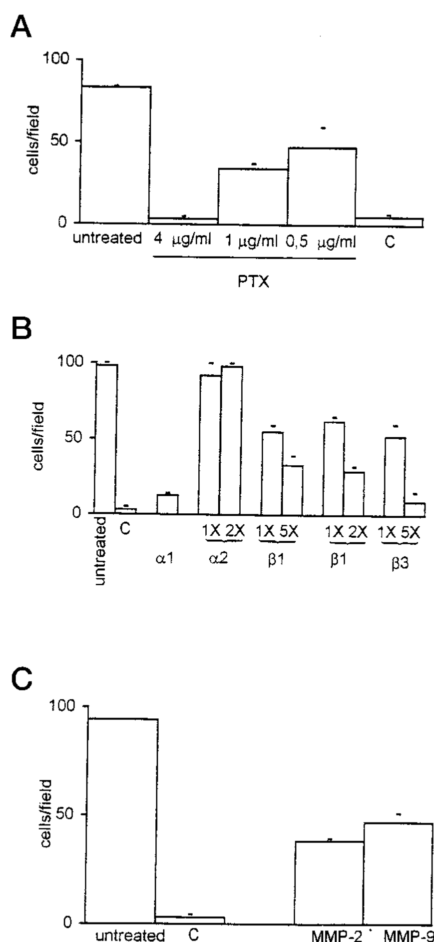

FIG. 2. **Requirement for chemoinduction of G_o/G_i protein activity, integrins, and MMP-2 and -9 function.** A standard Boyden assay with CM and EA hy926 is shown. *A*, cells were treated with the indicated concentrations of pertussis toxin (*PTX*) during assay (6 h). *B*, chemotaxis was assayed in 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. 3*A*), 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

TABLE II
Heat lability, pH, and EDTA and reducing agent sensitivity of chemoattractant

The effect of pH on chemotactic activity was tested by the addition to CM, for 3 min under stirring, of NaOH or HCl in amounts predetermined to obtain the desired pH, followed by neutralization before use in Boyden chamber assay. CM was utilized directly after the addition of EDTA or β-mercaptoethanol. Heat lability was tested by setting 1 ml of CM in a preheated bath for 15 min under stirring, followed by chilling in ice. S.D. values are less than 5%.

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

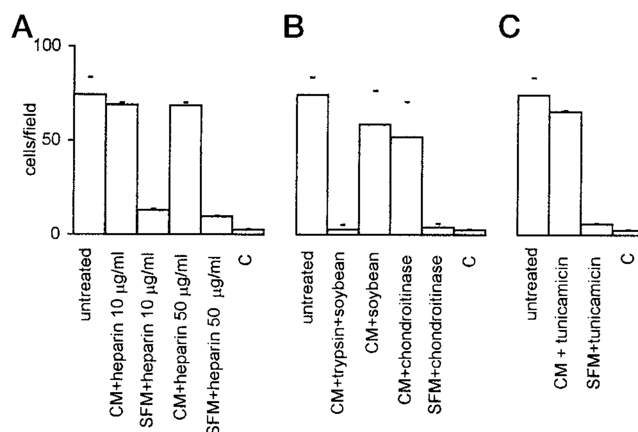

FIG. 3. **Chemotaxis induced by CM is unaffected by the addition of heparin and is independent from glycosylation of osteoblast proteoglycans.** A standard Boyden assay with CM and EA hy926 is shown with the addition of heparin at different concentrations to CM (*A*), with CM pretreated with trypsin followed by soybean trypsin inhibitor or with chondroitinase (*B*), or CM collected during incubation of osteoblasts with tunicamicin (*C*). All *panels* also show the results obtained when SFM was added with heparin, soybean trypsin inhibitor, chondroitinase, or tunicamicin to serve as control for lack of effect of these substances on chemotaxis. 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.

of 0.5 M 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 M NaCl. The average yield of protein after the purification was 1.5% of the starting amount. Fig. 4*A* illustrates the purification by heparin-Sepharose chromatography

Downloaded from http://www.jbc.org/ by guest on July 25, 2018

TABLE III
Fractionation of osteoblasts CM by molecular size and chromatographic affinity on heparin-Sepharose

Fractionation of CM by molecular size was obtained by centrifugation in Centriplus tubes fitted with molecular sieve filters of the indicated molecular size cut. Filtration was from higher to lower molecular size filter (left). Heparin-Sepharose chromatography of CM is shown on the right. Elution was by steps with increasing concentrations of NaCl in Tris-HCl (50 mM), pH 7.5. Fractions were tested by standard Boyden chamber assay, and numbers refer to migrated cells/field ± S.D. (both left and right).

Molecular size	Cells/field	NaCl	Cells/ field
		M	
<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		

of a preparation of CM metabolically labeled with Tran^{35}S-labeled amino acids, and Fig. 4*B* 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 α_1 chain, starting at position 1208, and the second sequence corresponds to rat type I procollagen α_2 chain, starting at position 1126 (Fig. 4*C*). 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 α_1 and α_2 chain carboxyl termini 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 α_1 and one α_2 carboxyl-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 carboxyl propeptide of type I collagen (C3). In agreement with the identification of the chemoattractant 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 α_1 and α_2 carboxyl-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.

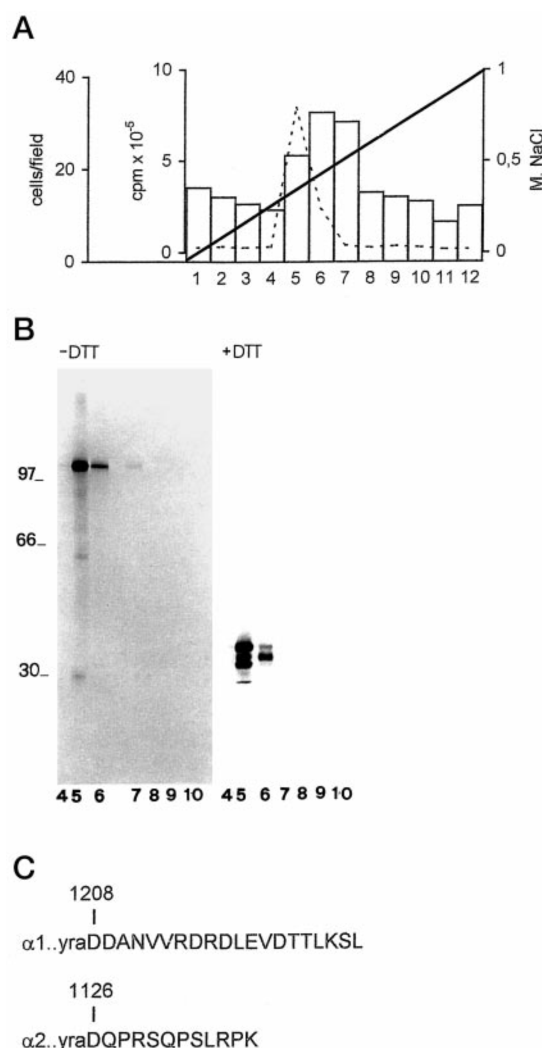

FIG. 4. **Purification of the chemoattractant from CM.** *A*, standard Boyden assay with EA hy926 of fractions recovered from a heparin-Sepharose column loaded with the >50-kDa fraction from CM metabolically labeled with Tran^{35}S-labeled methionine. The *white bars* indicate the cells migrated/field, the *interrupted line* indicates the cpm, and the *continuous line* indicates the concentration of the eluting NaCl gradient. Fraction numbers are indicated *below*. *B*, the fractions (*4–10*, *below*) eluted from the heparin-Sepharose column were run unreduced or after reduction on 10% acrylamide-SDS gels. *Numbers* on the *left* refer to molecular weight markers. *C*, sequence of the purified proteins. The sequences identified are shown in *uppercase type*. The sequences of α_1 and α_2 rat procollagen type I preceding the starting site are shown in *lowercase type*, and the *numbers above* indicate the position within the sequence of procollagen chains.

DISCUSSION

We show that the purified carboxyl-terminal propeptide trimer of collagen type I plays a previously unsuspected biological role as chemoattractant toward endothelial cells. During osteogenesis *in vitro*, C3 molecules are generated as a by-product of collagen biosynthesis by mature osteogenic cells, and chemoattractant 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 chemoattractant for endothelial cells and tumor cells produced by ROB in primary cultures (8, 9). We also detected a similar pattern of chemoattractant expression by analysis of the chemotaxis of endothelial cells induced by CM collected during *in vitro* osteogenesis of

Downloaded from http://www.jbc.org/ by guest on July 25, 2018

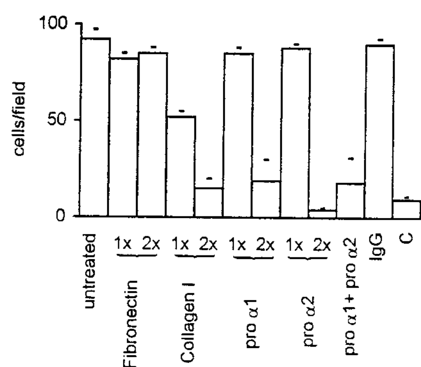

FIG. 5. **Antibodies against type I collagen and C procollagen chains inhibit chemoinduction.** Standard Boyden assays were performed with EA hy926 in the presence of two concentrations each of polyclonal antibodies against fibronectin, type I collagen, α_1 and α_2 carboxyl-terminal propeptides of type I collagen, and IgG (54 μg/ml) as control, added to the CM compartment. *Error bars* indicate S.E.

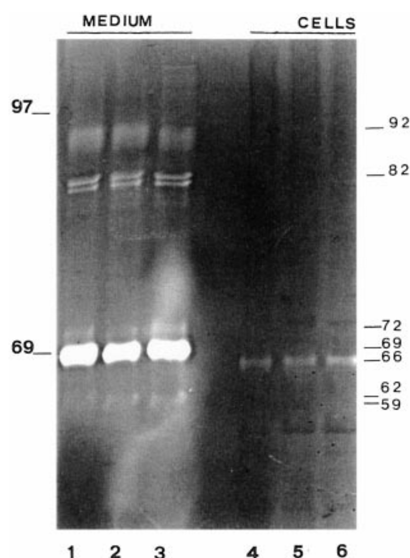

FIG. 6. **Chemoinduction by osteoblast CM and purified C3 is not dependent on induction of gelatinases in EA hy926.** Gelatin zymograms of conditioned media (*lanes 1–3*) and lysates (*lanes 4–6*) of EA hy926 were collected after exposure, for 6 h in Boyden chamber, to osteoblast chemoattractant CM (*lanes 1* and *4*), to serum-free medium (*lanes 2* and *5*), and to purified chemoattractant (C3, *lanes 3* and *6*). *Right*, molecular size of MMPs; *left*, molecular weight markers.

normal and transformed human osteoblasts.[2]

The chemotactic response of endothelial cells requires the activation of G_o/G_i-proteins, the functionality of integrins α_1, β_1, and β_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 β_1 integrin receptors are likely candidates.

$\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins were shown to interact with collagen carboxyl 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 down-regulated in presence of high calcium levels (25). Synthesis of both integrins is up-regulated by VEGF in endothelial cells (26). We demonstrate an inhibitory effect on migration of endothelial cells using antibodies to α_1 and β_1 integrins, not present with antibodies against α_2 integrin. Since we have utilized only one blocking monoclonal antibody against α_2 integrin, this last finding is not conclusive about the relevance of α_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 $\alpha_1\beta_1$ integrin to cross into the gelatin coating the filters, and further testing is required to discriminate between these possibilities.

The expression of β_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 β_3 integrin (27, 28). We do not presently know what role β_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 depose 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-β1 (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 deposed by osteoblasts and periosteal cells at this site. Production of C3 might represent

[2] D. Palmieri, V. Ulivi, and P. Manduca, unpublished observation.

Downloaded from http://www.jbc.org/ by guest on July 25, 2018

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.

Acknowledgments—We gratefully acknowledge the many colleagues who made possible this work by kindly providing cell lines and reagents.

REFERENCES

1. Folkman, J., and Shing, Y.(1992) *J. Biol. Chem.* **267,** 10931–10934
2. Montesano, R. (1992) *Eur. J. Clin. Invest.* **22,** 504–515
3. Klagsbrun, M., and D'Amore, P. (1991) *Annu. Rev. Physiol.* **53,** 217–239
4. Gerber, H. P., Vu, T. H., Ryan, A. M., Kowalski, J., Werb, Z., and Ferrara, N. (1999) *Nat. Med.* **5,** 623–627
5. Vu, T. H., Shipley, J. M., Bergers, G., Berger, J. E., Helms, J. A., Hanahan, D., Shapiro, S. D., Senior, R. M., and Werb, Z. (1998) *Cell* **93,** 411–422
6. Stringa, E., Filanti, C., Giunciuglio, D., Albini, A., and Manduca, P. (1995) *Bone* **16,** 663–670
7. Manduca, P., Palermo, C., Caruso, C., Brizzolara, A., Sanguineti, C., Filanti, C., and Zicca, A. (1997) *Bone* **21,** 31–36
8. Giunciuglio, D., Cai, T., Filanti, C., Manduca, P., and Albini, A. (1995) *Cancer Lett.* **97,** 69–74
9. Festuccia, C., Giunciuglio, D., Guerra, F., Villanova, I., Angelucci, A., Manduca, P., Teti, A., Albini, A., and Bologna, M. (1999) *Oncol. Res.* **11,** 17–31
10. Hojima, Y., Van der Rest, M., and Prockop, D. (1985) *J. Biol. Chem.* **260,** 15996–16003
11. Kessler, E., Takahara, K., Biniaminov, L., Brusel, M., and Greenspan, D. S. (1996) *Science* **271,** 360–362
12. O'Reilly, M. S., Boehm, T., Shing, Y., Fukai, N., Vasios, G., Lane, W. S., Flynn, E., Birkhead, J. R., Olsen, B. R., and Folkman, J. (1997) *Cell* **88,** 277–285
13. Alini, M., Matsui, Y., Dodge, G. R., Poole, A. R., Alini, M., Matsui, Y., Dodge, G. R., and Poole, A. R. (1992) *Calcif. Tissue Int.* **50,** 327–335
14. Van der Rest, M., Rosenberg, L. C., Olsen, B. R., and Poole, A. R. (1986) *Biochem. J.* **237,** 923–925
15. Ramchandran, R., Dhanabal, M., Volk, R., Waterman, M. J., Segal, M., Lu, H., Knebelmann, B., and Sukhatme, V. P. (1999) *Biochem. Biophys. Res. Commun.* **255,** 735–739
16. Kamphaus, G. D., Colorado, P. C., Panka, D. J., Hopfer, H., Ramchandran, R., Torre, A., Maeshima, Y., Mier, J. W., Sukhatme, V. P., and Kalluri, R. V. (2000) *J. Biol. Chem.* **275,** 1209–1215
17. Dejana, E., Languino, L. R., Polentarutti, N., Balconi, G., Ryckewaert, J. J., Larrieu, M. J., Donati, M. B., Mantovani, A., and Marguerie, G. (1985) *J. Clin. Invest.* **75,** 11–18
18. Parent, C. A., Blacklock, B. J., Froehlich, W. M., Murphy, D. B., and Devreotes, P. N. (1998) *Cell* **95,** 81–91
19. Davies, D., Tuckwell, D. S., Calderwood, D. A., Weston, S. A., TakiGawa, M., and Humphries, M. J. (1997) *Eur. J. Biochem.* **246,** 274–282
20. Weston, S. A., Hulmes, D. J. S., Mould, A. P., Watson, R. B., and Humphries, M. J. (1994) *J. Biol. Chem.* **269,** 20982–20986
21. Pozzi, A., Wary, K. K., Giancotti, F. G., and Gardner, H. A. (1998) *J. Cell Biol.* **142,** 587–594
22. Broberg, A., and Heino, J. (1996) *Exp. Cell Res.* **228,** 29–35
23. Riikonen, T., Westermarck, J., Koivisto, L., Broberg, A., Kahari, V. M., and Heino, J. (1995) *J. Biol. Chem.* **270,** 13548–13552
24. Langholz, O., Rockel, D., Mauch, C., Kozlowska, E., Bank, I., Krieg, T., and Eckes, B. (1995) *J. Cell Biol.* **131,** 1903–1915
25. Dickson, K. S., Walsh, J. J., and Santoro, S. A. (1997) *J. Biol. Chem.* **272,** 7661–7668
26. Senger, D. R., Claffey, K. P., Benes, J. E., Perruzzi, C. A., Sergiou, A. P., and Detmar, M. (1997) *Proc. Natl. Acad. Sci.* **94,** 13612–13617
27. Brooks, P. C., Clark, R. A., and Cheresh, D. A. (1994) *Science* **264,** 569–571
28. Soldi, R., Mitola, S., Strasly M., Defilippi, P., Tarone, G., and Bussolino, F. (1999) *EMBO J.* **18,** 882–892
29. Hiraoka, N., Allen, E., Apel, I. J., Gyetko, M. R., and Weiss, S. J. (1998) *Cell* **95,** 365–377
30. Yoshitake, H., Denhardt, D. T., and Noda, M. (1999) *J. Bone Mineral Res.* **14,** Suppl. 1, 1004
31. Davis, S., Aldrich, T. H., Jones, P. F., Acheson, A., Compton, D. L., Jain, V., Ryan, T. E., Bruno, J., Radziejewski, C., Maisonpierre, P. C., and Yancopoulos, G. D. (1996) *Cell* **87,** 1161–1169
32. Festuccia, C., Bologna, M., Gravina, G. L., Guerra, F., Angelucci, A., Villanova, I., Millimaggi, D., and Teti, A. (1999) *Int. J. Cancer* **81,** 395–403
33. Aycock, R. S., Raghow, W. R., Strickilin, G. P., Seyer, J. M., and Kanga, M. (1986) *J. Biol. Chem.* **261,** 14355–14360
34. Wu, C. H.,Walton, C. M., and Wu, G. Y. (1991) *J. Biol. Chem.* **266,** 2983–2987
35. Gardner, H., Broberg, A., Pozzi, A., Laato, M., and Heino, J. (1999) *J Cell Sci.* **112,** 263–272
36. Choglay, A. A., Purdom, I. F., and Hulmes, D. J. S. (1993) *J. Biol. Chem.* **268,** 6107–6114

Downloaded from http://www.jbc.org/ by guest on July 25, 2018

Trimer Carboxyl Propeptide of Collagen I Produced by Mature Osteoblasts Is Chemotactic for Endothelial Cells
Daniela Palmieri, Laura Camardella, Valentina Ulivi, Gaetana Guasco and Paola Manduca

J. Biol. Chem. 2000, 275:32658-32663.
doi: 10.1074/jbc.M002698200 originally published online August 2, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M002698200

Alerts:

- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 36 references, 17 of which can be accessed free at
http://www.jbc.org/content/275/42/32658.full.html#ref-list-1

Downloaded from http://www.jbc.org/ by guest on July 25, 2018