

Transferrin Promotes Endothelial Cell Migration and Invasion: Implication in Cartilage Neovascularization

Mariella F. Carlevaro,* Adriana Albini,* Domenico Ribatti,^{||} Chiara Gentili,* Roberto Benelli,* Silvia Cermelli,* Ranieri Cancedda,*[‡] and Fiorella Descalzi Cancedda*[§]

*Istituto Nazionale per la Ricerca sul Cancro, Centro di Biotecnologie Avanzate and [‡]Dipartimento di Oncologia Clinica e Sperimentale, Università di Genova, Genova, Italy; [§]Istituto Internazionale di Genetica e Biofisica, Consiglio Nazionale delle Ricerche, Napoli, Italy; and ^{||}Istituto di Anatomia Umana Normale, Istologia ed Embriologia, Università di Bari, Bari, Italy

Abstract. During endochondral bone formation, avascular cartilage differentiates to hypertrophic cartilage that then undergoes erosion and vascularization leading to bone deposition. Resting cartilage produces inhibitors of angiogenesis, shifting to production of angiogenic stimulators in hypertrophic cartilage. A major protein synthesized by hypertrophic cartilage both in vivo and in vitro is transferrin. Here we show that transferrin is a major angiogenic molecule released by hypertrophic cartilage. Endothelial cell migration and invasion is stimulated by transferrins from a number of different sources, including hypertrophic cartilage. Checkerboard analysis demonstrates that transferrin is a chemotactic and chemokinetic molecule. Chondrocyte-conditioned media show similar properties. Polyclonal anti-transferrin antibodies completely block endothelial cell migration and invasion induced by purified transferrin and inhibit the activity produced by

hypertrophic chondrocytes by 50–70% as compared with controls. Function-blocking mAbs directed against the transferrin receptor similarly reduce the endothelial migratory response. Chondrocytes differentiating in the presence of serum produce transferrin, whereas those that differentiate in the absence of serum do not. Conditioned media from differentiated chondrocytes not producing transferrin have only 30% of the endothelial cell migratory activity of parallel cultures that synthesize transferrin.

The angiogenic activity of transferrins was confirmed by in vivo assays on chicken egg chorioallantoic membrane, showing promotion of neovascularization by transferrins purified from different sources including conditioned culture medium.

Based on the above results, we suggest that transferrin is a major angiogenic molecule produced by hypertrophic chondrocytes during endochondral bone formation.

THE regulation of angiogenesis is a key feature in many developmental and pathological processes (Folkman and Shing, 1992). Tumor angiogenesis, where resting endothelial cells are stimulated to proliferate, migrate, invade, and differentiate to form new vessels, is now considered a key target for controlling tumor growth and metastasis (Folkman, 1995). Growth plate cartilage is a very interesting tissue to investigate mechanisms controlling angiogenesis. Cartilage is normally an avascular tissue, but, after differentiation to hypertrophic cartilage in the growth plate, invasion by blood vessels is absolutely required for bone formation to proceed. Angiogenic inhibitors have been purified from both resting and proliferative cartilage (Moses et al., 1990, 1992; Pepper et al., 1991). In contrast, angiogenic activity has been described to be associated with mineralized hypertrophic chondrocytes in vitro (Brown and McFarland, 1992). We have recently shown

(Descalzi Cancedda et al., 1995) that chick embryo chondrocytes undergoing in vitro differentiation from prechondrogenic to hypertrophic cells in the absence of ascorbic acid constitutively express antiangiogenic activity. However, when the hypertrophic chondrocyte culture is supplemented with ascorbic acid, there exists formation of a mineralized tissue in vitro similar to that found in vivo in calcified hypertrophic cartilage, and a switch to production of angiogenic activity. Therefore, the interaction of the cells with the surrounding organized extracellular matrix is an absolute requirement for the expression of the angiogenic activity.

We have also shown that in vitro differentiated hypertrophic chondrocytes cultured in the presence of ascorbic acid synthesize a large amount of a 69-kD protein identified as transferrin (Gentili et al., 1993). The presence of transferrin in vivo was demonstrated by immunohistochemistry and by in situ hybridization in the embryonic hypertrophic cartilage. The presence of the transferrin receptor on the surface of hypertrophic chondrocytes and, at particularly high levels, on the surface of adjacent undiffer-

Address all correspondence to Fiorella Descalzi Cancedda, Centro di Biotecnologie Avanzate, Largo Rosanna Benzi, 10, 16132 Genova, Italy. Tel.: (39) 10-5737392. Fax: (39) 10-5737405. e-mail: Cancedda@sirio.cba.unige.it

entiated cells in the diaphyseal collar was demonstrated. Based on these data, a possible autocrine and paracrine role of the transferrin in the developing bone was suggested (Gentili et al., 1994). Transferrins are a heterogeneous group of proteins caused by variations in the polypeptide chain, glycanation, and iron content. The major role of transferrins is iron transport; however, transferrin has other biological activities and has been found to be important for growth and differentiation of several cell types. These activities include a role as a mitogenic (Trowbridge and Omary, 1981), neurotrophic (Aizenman et al., 1986), and myotrophic (Li et al., 1982) factor as well as embryo morphogenesis (Ekblom et al., 1983; Partanen et al., 1984). In addition, a paracrine action of transferrin has also been shown in studies investigating the growth of metastatic tumors. The transferrin released by lung cells stimulates the growth of lung metastasizing tumor cells (Cavanaugh and Nicolson, 1991). The transferrin produced by brain cells plays a major role in supporting proliferation of melanoma cells in brain metastases (Menter et al., 1995).

Since the expression of transferrin in differentiating chondrocytes correlates with the switch to an angiogenic-inducing phenotype, we have tested the hypothesis of a paracrine action of the transferrin in promoting neovascularization of growth plate cartilage.

Materials and Methods

Cell Culture

Chondrocyte cell culture methods have been extensively described elsewhere (Castagnola et al., 1986; Descalzi Cancedda et al., 1992). Briefly, 6-d-old chick embryo tibiae were digested, and the freshly dissociated chondrocytes were plated on culture dishes. Dedifferentiated cells were maintained in Coon's modified F12 medium with 10% FCS and cultured in adhesion for the following 3 wk. Cells were then transferred to suspension culture on agarose-coated dishes where they resumed differentiation and in 3–4 wk formed a nearly homogeneous population of single isolated hypertrophic chondrocytes. To eliminate residual cell clusters, cells were filtered through a nylon filter. In vitro–formed cartilage was obtained by the culture of these cells in suspension in the presence of 100 µg/ml ascorbic acid and 10 mM β-glycerophosphate.

Alternatively, cells were cultured in a serum-free medium (Quarto et al., 1992). Dedifferentiated cells were suspended in Coon's modified F12 medium supplemented with T₃ (10⁻¹⁰ M), insulin (60 ng/ml), and Dex (10⁻¹⁰ M). Fully differentiated hypertrophic chondrocytes were obtained after 1 wk in culture.

AIDS-KS cells (Albini et al., 1992) were cultured in RPMI 1640/DME (1:1) supplemented with 10% FCS (Gibco Ltd., Paisley, Scotland). EAhy 926 hybrid endothelial cells were the kind gift of Dr. C.J. Edgell (University of North Carolina, Chapel Hill) and were maintained in DME containing 10% FCS (Edgell et al., 1983).

Preparation of Conditioned Medium from Cultured Chondrocytes and Kaposi Sarcoma Cells

Hypertrophic chondrocytes in different culture conditions were rinsed twice with PBS and maintained in F12 medium for 24 h (5 ml for 100-mm dish) without any addition. This medium was collected and used for chemotaxis assay. Metabolic labeling of the cell culture after incubation with F12 alone confirmed that the protein synthesis remained unchanged in quality and quantity with respect to the previous culture conditions.

As positive controls for angiogenesis, conditioned media from Kaposi sarcoma (KS)¹ cells were used (Albini et al., 1992, 1994; Salahuddin et al., 1988; Thompson et al., 1991). KS cells at 80% confluency were rinsed twice

and incubated for 24 h with serum-free medium (8 ml per T75 flask) before conditioned media (CM) collection (KS-CM).

Conditioned media from both cultures were centrifuged for 10 min at 3,000 rpm to eliminate cell debris and stored at –20°C.

DNA Measurement

DNA content of cultured cells was determined to insure that corresponding concentrations of conditioned media were used. Cell layers were collected in 0.01% SDS in PBS and digested overnight at 50°C with proteinase K (Sigma Chemical Co., St. Louis, MO) at a concentration of 100 µg/ml in 10 mM Tris-HCl, pH 7.8, and 5 mM EDTA.

0.1 ml of each sample was added to 2 ml dye solution (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.1 M NaCl, and 0.1 mg/ml Hoechst 33258 [Sigma Chemical Co.], and DNA determination was performed in a DNA fluorimeter from Hoefer Scientific Instruments (San Francisco, CA).

Chemoinvasion Assay

The Boyden chamber chemoinvasion assay was carried out with slight modifications, as previously described (Albini et al., 1987). Matrigel, a mixture of basement membrane components obtained as an Englebreth-Holm-Swarm murine sarcoma extract (Kleinman et al., 1986), was layered evenly on polycarbonate filters (12-µm pore size, PVP-free; Nucleopore, Concorezzo, Italy) at a concentration of 20–25 µg per filter, air dried, and then reconstituted with medium into a solid gel.

Conditioned media from KS cells or chondrocytes were placed in the lower compartment of Boyden chamber and used as chemoattractants at a concentration proportional to the DNA content of cell layers.

In some experiments, the chemoattractant was transferrin from various sources: apotransferrin from human plasma (Sigma Chemical Co.), ovotransferrin (conalbumin) from chicken egg white (Sigma Chemical Co.), and ovotransferrin purified from chicken chondrocyte-conditioned medium, as previously described (Gentili et al., 1993). Conditioned media used in the different experiments presented in Results were derived from different primary cultures of hypertrophic chondrocytes.

We have previously demonstrated that EAhy 926 hybrid endothelial cells and human umbilical vein endothelial cells behaved similarly when the chemotactic activity of unfractionated medium conditioned by hypertrophic chondrocytes was tested (Descalzi Cancedda et al., 1995). Based on these results, EAhy 926 hybrid endothelial cells were used for the experiments reported in the present work. Endothelial cells (1.3 × 10⁵ cells per chamber) were placed in the Boyden chamber's upper compartment after harvesting with trypsin and washing with serum-free DME.

Standard assay conditions were 6 h at 37°C in 5% CO₂. After mechanical removal of the cells remaining on the upper surface of the filter, cells that migrated to the undersurface were stained (Toluidine blue; Sigma Chemical Co.) and quantitated microscopically. Five to ten random fields were counted on each filter. Assays were performed in triplicate and repeated at least twice. When antibodies were used, chemoattractants with and without antibodies and controls were previously incubated overnight at 4°C.

Chemotaxis Assay

Chemotaxis assays were performed in the same way as those of chemoinvasion, with the only difference being that the filters were not coated with Matrigel but with gelatin (type A; Sigma Chemical Co.) at a concentration of 5 µg/ml as an attachment substrate.

In some experiments, chemoattractants (conditioned media or transferrins) were mixed with the cells in the upper compartment (checkerboard analysis).

When antibodies were used, chemoattractants with and without antibodies and controls were previously incubated overnight at 4°C.

Cell Metabolic Labeling and SDS-PAGE Analysis of Proteins

Cells were labeled with [³⁵S]methionine, as described by Descalzi Cancedda et al. (1992). Briefly, cultured cells were washed in PBS and incubated for 2 h at 37°C in methionine-free Coon's modified F12 medium with 100 µg/ml ascorbic acid. [³⁵S]Methionine was then added and incubation continued for an additional 2 h. Aliquots of culture media were run for protein analysis on 15% SDS-PAGE in nonreducing conditions. Immunoprecipitation of specific proteins was performed as previously described (Descalzi Cancedda et al., 1992).

1. Abbreviations used in this paper: bFGF, basic FGF; CAM, choriallantoic membrane; CM, conditioned medium; KS, Kaposi sarcoma.

Western Blot Analysis

Cell layers were lysed with 0.1% SDS in PBS. Aliquots of samples were loaded on a 15% SDS polyacrylamide gel and electrophoresis was performed in reducing conditions. After electrophoresis, the gel was blotted to a BA85 nitrocellulose membrane (Schleicher & Schuell GmbH, Dassel, Germany) according to the procedure described by Towbin et al. (1979).

The blot was saturated overnight with 5% skim milk powder (Merck Biochemica, Darmstadt, Germany) in TTBS buffer (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.05% Tween-20) and washed several times with TTBS; it was then incubated (for 3 h at room temperature) with the goat polyclonal antibody directed against the human placental transferrin receptor, made monospecific by adsorption on transferrin to avoid cross-reactivity (a gift from Dr. H.H. Sussman, Stanford University School of Medicine, Stanford, CA).

After further washes, detection was performed by a biotin-conjugated anti-goat IgG (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) and avidin-HRP (Jackson Immunoresearch Laboratories, Inc.), using 4-chloro-1-naphthol (Merck Biochemica) as substrate.

Chorioallantoic Membrane Assay

Fertilized White Leghorn chicken eggs (30 per group) were incubated under conditions of constant humidity at a temperature of 37°C. On the third day of incubation, a square window was opened in the egg shell after removal of 2–3 ml of albumen so as to detach the developing chorioallantoic membrane from the shell. The window was sealed with a glass of the same dimension, and the eggs were returned to the incubator.

Embryonic chorioallantoic membranes (CAMs) were treated at day 8 with (a) ovotransferrin purified from chicken hypertrophic chondrocyte-conditioned medium (1 µg); (b) ovotransferrin from chicken egg white (Sigma Chemical Co.) (1 µg); (c) apotransferrin from human plasma (Sigma Chemical Co.) (1 µg); and (d) concentrated (3 µl) and not concentrated (3 µl) conditioned medium from cultured hypertrophic chondrocytes adsorbed on 1-mm³ sterilized gelatin sponge (Gelfoam; Upjohn Company, Kalamazoo, MI). Sponges were implanted on top of growing CAM under sterile conditions, within a laminar-flow hood. Sponges containing vehicle alone (PBS) and a stimulator of blood vessel formation, namely basic FGF (bFGF; 1 µg), were used as negative and positive controls, respectively.

CAMs were examined daily until day 12 and photographed in ovo under a stereomicroscope SR equipped with the Camera System MC 63 (Zeiss, Oberkochen, Germany). At day 12, CAMs were processed for light microscopy. Briefly, the embryos and their membranes were fixed in ovo in Bouin's fluid. The sponges and the underlying and immediately adjacent CAM portions were removed and processed for embedding in paraffin. 8-µm serial sections were cut according to a plane parallel to the surface of the CAM and stained with a 0.5% aqueous solution of Toluidine blue (Merck Biochemica) and observed under a Leitz-Dialux 20 light microscope (Leitz, Wetzlar, Germany).

At day 12, some CAMs were also injected intravascularly with India ink according to Olivo et al. (1992).

Quantitation of the Angiogenic Response

The angiogenic response was assessed by a planimetric method of point counting (Elias and Hyde, 1983; Ribatti et al., 1995). Briefly, every third section within 30 serial slides from an individual specimen was analyzed by a 144-point mesh inserted in the eyepiece of a Leitz-Dialux 20 photomicroscope. Six randomly chosen microscopic fields of each section were evaluated at ×250 magnification for the total number of the intersection points that were occupied by vessels transversally cut (diameter ranging from 3–10 µm). Mean values ± SD for vessel counts were determined for each analysis.

The vascular density was indicated by the final mean number of the occupied intersection points. The statistical significance of differences between the mean values of the intersection points in the experimental CAMs and control ones was determined by the *t* test for unpaired data.

Results

Enhancement of Endothelial Cell Migration and Invasion by Purified Transferrins

The production of transferrins at high levels by differenti-

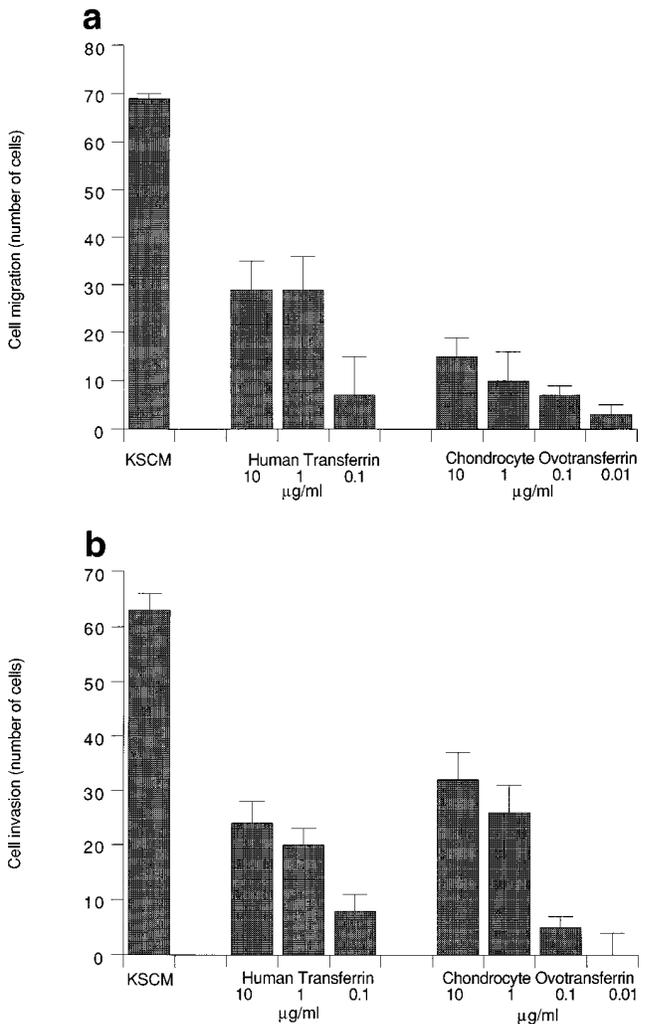


Figure 1. Chemotactic (a) and chemoinvasive (b) responses of vascular EAhy 926 cells to human transferrin and chicken ovotransferrin purified from chondrocyte-conditioned medium. The factors were dissolved in F12 medium. F12 medium alone was used as control for background random migration that has been subtracted. Assays were performed in triplicate and repeated at least twice. Five fields were counted on each triplicate filter. Bars indicate SD. A typical experiment is shown. KSCM, control conditioned medium from Kaposi sarcoma cells.

ating hypertrophic chondrocytes, and the correlation of transferrin with angiogenic activity produced by these cells, led us to test if transferrin could induce migration and invasion of endothelial cells. Commercial human transferrin, purified from serum, and chicken ovotransferrin, purified from chondrocyte-conditioned culture medium, induced chemotactic migration and basement membrane invasion by vascular cells in a Boyden chamber assay (Fig. 1, a and b). A dose-dependent response was observed with a maximal migration at 10 µg/ml. Both forms of commercial human transferrin, holotransferrin and apotransferrin, were tested with identical results.

Human transferrin, chicken egg white ovotransferrin, and ovotransferrin purified from hypertrophic chondrocyte-conditioned culture medium (all at 10 µg/ml) were compared for induction of endothelial cell migration (Fig. 2).

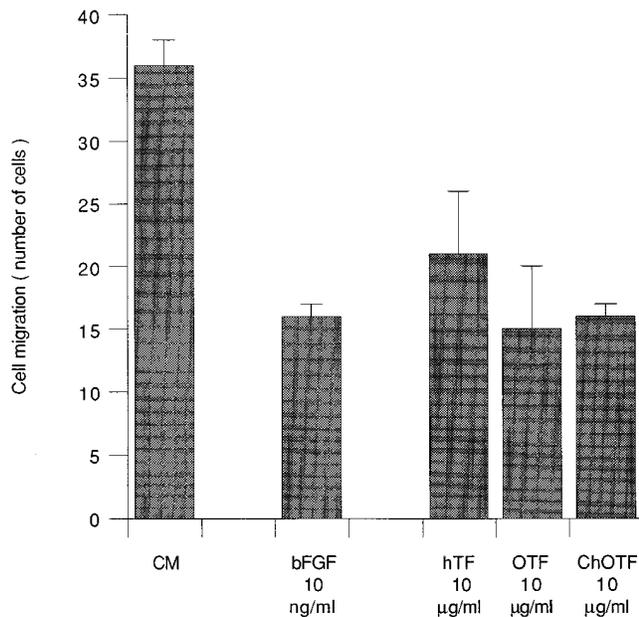


Figure 2. Comparison of chemotactic responses of vascular cells to hypertrophic chondrocyte-conditioned culture medium, to human transferrin (*hTF*), to chicken egg white ovotransferrin (*OTF*), to ovotransferrin, purified from chondrocyte-conditioned medium (*ChOTF*), and to human bFGF. Conditioned medium (*CM*) was obtained from hypertrophic chondrocytes grown in suspension for ~3 wk in the presence of ascorbic acid. F12 medium with 0.1% BSA was analyzed as control to verify background random migration. Assays were performed and analyzed as in Fig. 1 and repeated at least twice. Bars indicate SD. A typical experiment is shown.

Recombinant human bFGF (10 ng/ml), as a positive control, and conditioned medium from hypertrophic chondrocytes in suspension with ascorbic acid for 21 d were also tested. All the factors showed comparable effects on the stimulation of endothelial cell migration (Fig. 2). When ovotransferrin levels in conditioned media from four randomly chosen cultures were determined by a Western blotting assay, values ranging from 1–10 µg/ml were obtained. This is in agreement with the maximal response to purified transferrin observed and the variation in the activity of individual conditioned media preparations.

Checkerboard analysis performed both on human transferrin (Table I) and on ovotransferrin (not shown) revealed a chemotactic activity enhancement. Comparable levels of endothelial cell migration were observed for human transferrin (10/1/0.1 µg/ml) as compared to bFGF (10/1/0.1 ng/ml) by checkerboard analysis (data not shown). Similar results were obtained when checkerboard analysis was performed on unfractionated conditioned medium from hypertrophic chondrocytes grown in suspension in the presence of ascorbic acid (Table II).

Antibodies against Transferrin Inhibit Endothelial Cell Migration and Invasion in Boyden Chamber Assay

Conditioned culture medium from hypertrophic chondrocytes and purified ovotransferrin was tested for chemotactic and chemoinvasive activity in the absence and presence of affinity-purified antibodies against ovotransferrin (Fig. 3,

Table I. Checkerboard Analysis of Human Transferrin in F12

Lower chamber	Upper chamber		
	SFM	hTF 0.1 µg/ml	hTF 10 µg/ml
SFM	22 ± 5*	29 ± 3	23 ± 5
hTF 0.1 µg/ml	35 ± 3*	30 ± 7	24 ± 2
hTF 10 µg/ml	42 ± 7*	34 ± 2	32 ± 8

Different concentrations of human transferrin in F12 (10/0.1/0 µg/ml) were added to the upper or the lower chamber or both. Data were collected and analyzed as described in Materials and Methods.

*The dose dependence is shown in the first orthogonal lane.

SFM, serum-free F12 medium, corresponding to 0 µg/ml hTF.

a and *b*). These antibodies strongly, but not completely, inhibited chemotaxis and chemoinvasion induced by hypertrophic chondrocyte-conditioned medium and completely inhibited both activities induced by the purified ovotransferrin. Nonspecific antibodies (purified Ig from murine myeloma; Sigma Chemical Co.) at equivalent concentrations did not show any inhibitory effect (data not shown). These data indicate that ovotransferrin in the conditioned culture medium plays a major role in inducing migration and invasion of endothelial cells, but that additional factors which contribute to the response are present in the medium.

Regulated Expression of Ovotransferrin and Angiogenic Activity in Hypertrophic Chondrocytes Cultured in a Serum-free Medium

We have previously reported that in the absence of FCS, dedifferentiated chondrocytes undergo a differentiation in vitro to single hypertrophic chondrocytes when they are transferred in suspension culture in the presence of thyroid hormone, insulin, and dexamethasone (Quarto et al., 1992). Hypertrophic chondrocytes obtained using this serum-free culture system were supplemented with ascorbic acid and split in two subcultures. One subculture was additionally supplemented with 10% FCS, while the other control subculture was not. In both subcultures, due to the presence of ascorbic acid, the hypertrophic chondrocytes organized an extracellular matrix and rapidly aggregated, giving rise to a tissue strongly resembling hypertrophic cartilage as partially previously reported (Descalzi Cancedda et al., 1995). Nevertheless, metabolic labeling established that differences existed in the two subcultures with respect to proteins synthesized and released in the culture

Table II. Checkerboard Analysis of Conditioned Medium from Cultures of Hypertrophic Chondrocytes in Suspension with Ascorbic Acid for ~3 wk

Lower chamber	Upper chamber			
	SFM	10% CM	20% CM	50% CM
SFM	22 ± 2* [‡]	18 ± 2	17 ± 1	17 ± 4
10% CM	31 ± 6*	26 ± 3 [‡]	25 ± 4	22 ± 4
20% CM	36 ± 4*	40 ± 6	30 ± 6 [‡]	27 ± 3
50% CM	44 ± 6*	41 ± 4	36 ± 3	32 ± 6 [‡]

F12 culture medium was added with increasing volumes (0/10/20/50%) of CM. The different dilutions were added to the upper or the lower chamber or both. Data were collected and analyzed as described in Materials and Methods.

*The dose dependence is shown in the first orthogonal lane.

[‡]The dose dependence of the chemokinetic component is indicated on the diagonal.

SFM, serum-free medium, corresponding to 0% CM.

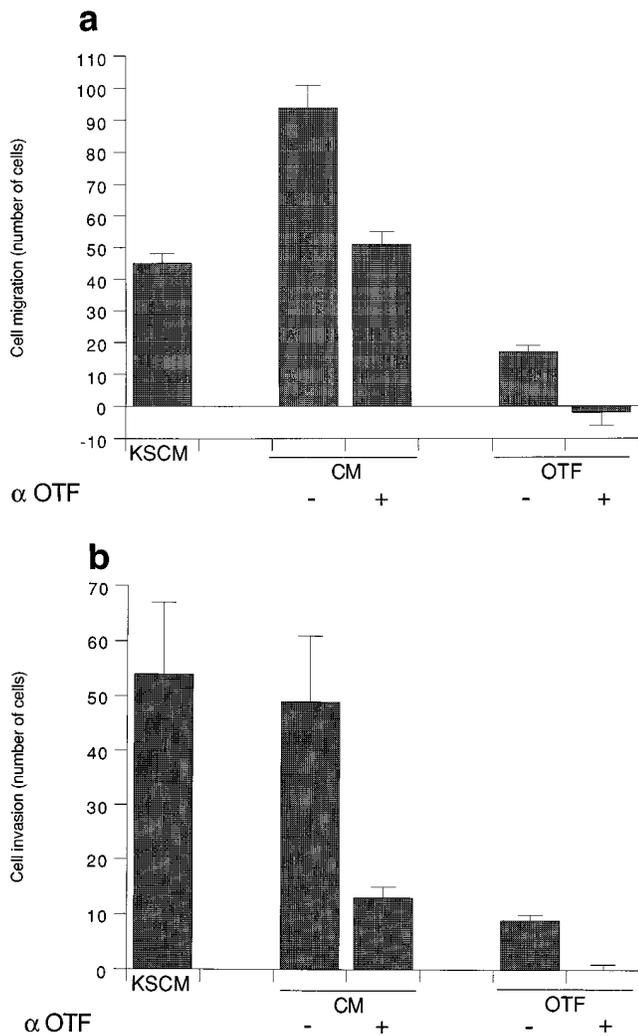


Figure 3. Chemotactic (a) and chemoinvasive (b) responses of vascular cells to hypertrophic chondrocyte-conditioned culture medium and to ovotransferrin, purified from chondrocyte-conditioned medium, and their inhibition by affinity-purified antibodies against ovotransferrin. Conditioned medium (CM) was obtained from hypertrophic chondrocytes grown in suspension for ~ 3 wk in the presence of ascorbic acid; ovotransferrin (OTF) was used at a concentration of 10 $\mu\text{g/ml}$. Analogous effects were obtained with a lower antibody concentration (10 $\mu\text{g/ml}$). Cell migration in the presence of antibodies alone in F12 was not significantly different from background random migration. Assays were performed and analyzed as in Fig. 1 and repeated at least twice on different culture media. Bars indicate SD. A typical experiment is shown. αOTF , experiments with antibodies against ovotransferrin at 50 $\mu\text{g/ml}$.

medium (Fig. 4). In particular, hypertrophic chondrocytes synthesized and secreted ovotransferrin only when the culture medium was additionally supplemented with FCS.

After 8 d, media in both subcultures were replaced for 24 h with the same fresh medium without ascorbic acid and FCS. These conditioned media from both subcultures were tested for chemotactic activity in the Boyden chamber assay. Both media gave a positive response, but to a different extent. The chemotactic activity in the medium from the subculture not supplemented with FCS (not containing ovotransferrin) showed chemotactic activity that

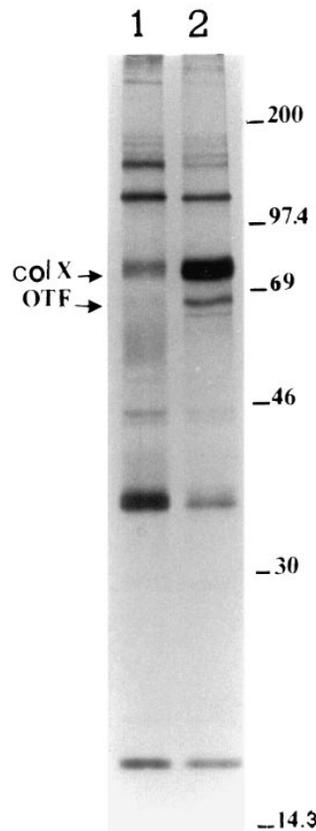


Figure 4. Nonreducing 15% SDS-PAGE of metabolically labeled proteins from conditioned medium of hypertrophic chondrocytes, after 8 d of culture in suspension with ascorbic acid. (Lane 1) Medium not supplemented with FCS. (Lane 2) Medium supplemented with FCS. (Arrows) Ovotransferrin (OTF) and collagen type X. OTF was identified by immunoprecipitation (not shown).

was about one-third of the activity present in the medium from the supplemented subculture (producing ovotransferrin) (Fig. 5). As expected, the activity present in the FCS-free subculture was not significantly affected by the addition of antibodies against ovotransferrin. The activity present in the medium from the supplemented, transferrin-producing subculture was reduced by the addition of the same antibodies to about one-third, i.e., to levels similar to that of the unsupplemented culture.

Blocking Antibodies Directed against the Transferrin Receptor Prevent Chemotactic Migration of Endothelial Cells

The presence of transferrin receptors on the membrane of EAhy 926 cells was demonstrated by Western blot analysis. (Fig. 6). Affinity-purified polyclonal antibodies directed against the transferrin receptor identified a single 92-kD band on Western blots of EAhy 926 cell extracts that corresponds to the molecular mass of the known transferrin receptor. When function-blocking mAbs directed against the transferrin receptor (42/6; Trowbridge and Lopez, 1982) were added in the Boyden chamber assay, the chemotactic response of endothelial cells induced by the hypertrophic chondrocyte-conditioned medium was significantly reduced (50%), while the chemotactic response induced by purified transferrins was completely inhibited (Fig. 7). Polyclonal antibodies directed against ovotransferrin gave identical results.

Effects of Transferrins on Endothelial Cell Proliferation

We investigated whether or not transferrins induce endo-

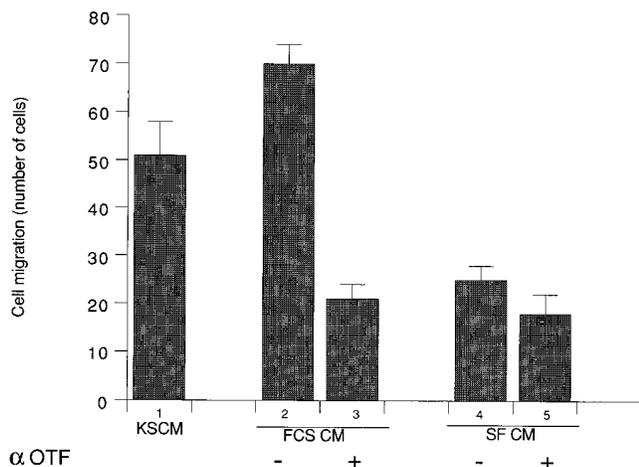


Figure 5. Chemotactic migration induced in vascular cells by hypertrophic chondrocyte-conditioned media, after 9 d in suspension with ascorbic acid, with or without FCS, and inhibition by affinity-purified antibodies against ovotransferrin. A population of hypertrophic chondrocytes grown in suspension for 3 wk was split, and the suspension culture continued in the presence of ascorbic acid, either in the presence or absence of FCS. Conditioned media were collected after 9 d of suspension; for the assays, CM were diluted proportionally to the DNA content of the cells. Antibody concentration was 10 μ g/ml. Cell migration in the presence of antibodies alone in F12 medium was not significantly different from background random migration. Assays were performed and analyzed as in Fig. 1 and repeated independently twice, on different culture media, with similar results. A typical experiment is shown. Bars indicate SD. *FCS CM*, conditioned medium from culture supplemented with FCS; *SF CM*, conditioned medium from serum-free cultures. α *OTF*, antibodies against ovotransferrin.

thelial cell proliferation, similar to the effect of other angiogenic growth factors. Immortalized EAhy 926 vascular cell growth was measured by the metabolic MTT assay (Carmichael et al., 1987), according to a published procedure (Albini et al., 1996). Endothelial cells were treated with human transferrin at different concentrations (10/1/0.1 μ g/ml) in their culture medium without FCS or supplemented with 0.1% FCS. Transferrin did not enhance endothelial cell proliferation; at each tested culture time (24/

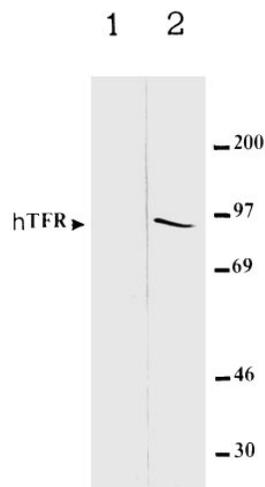


Figure 6. Western blot analysis for human transferrin receptor on EAhy 926 cells. Cells were scraped and lysed in 0.01% SDS in PBS. Aliquots of cell extracts were run on 15% SDS-PAGE in reducing conditions and blotted. The presence of the human transferrin receptor was detected with goat anti-human transferrin receptor antibodies. (Lane 1) negative control; (lane 2) anti-human transferrin receptor antibodies. (Arrow) Band with the expected 92-kD molecular mass of the transferrin receptor. (Left) Molecular mass standard migrations.

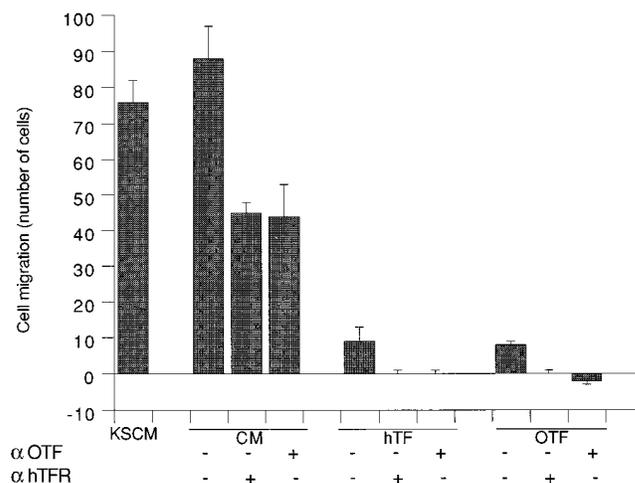


Figure 7. Chemotactic response by vascular cells and inhibition by the blocking mAb 42/6 against human transferrin receptor (α *hTFR*) and by polyclonal antibodies against ovotransferrin (α *OTF*). Chemoattractants were hypertrophic chondrocyte-conditioned culture medium, human transferrin, and ovotransferrin purified from chondrocyte-conditioned medium, as indicated. Antibody concentrations were 2.5 μ g/ml (α *hTFR*) and 10 μ g/ml (α *OTF*). Cell migration with antibodies alone in F12 was not significantly different from background random migration. Ovotransferrin (*OTF*) and human transferrin (*hTF*) were used at 10 μ g/ml. Assays were performed and analyzed as in Fig. 1 and repeated at least twice on different culture media. A typical experiment is shown. Bars indicate SD. *CM*, conditioned medium from hypertrophic chondrocytes.

48/72/96/144 h), the cells exhibited a doubling time similar to that observed with control medium alone. Cells in the presence of 10% FCS actively proliferated (data not shown).

In Vivo Assay (CAM) of the Angiogenic Activity of Transferrins and Hypertrophic Chondrocyte-conditioned Culture Medium

Angiogenic effects of transferrins of various origin and of hypertrophic chondrocyte-conditioned culture medium were investigated in vivo in the CAM assay. Embryonic chorioallantoic membranes at day 8 of incubation were implanted with sponges containing either ovotransferrin purified from chicken hypertrophic chondrocyte-conditioned medium, ovotransferrin from chicken egg white, apotransferrin from human plasma, or conditioned medium from cultured hypertrophic chondrocytes. Vehicle alone (PBS) and bFGF were used as negative and positive controls.

Table III. Quantitative Evaluation of the CAM Assay

Treatment	Total number	Positive reaction
		%
PBS	20	0
bFGF	20	80
Purified ovotransferrin	30	75
Chicken ovotransferrin	30	70
Human apotransferrin	30	70
Concentrated conditioned medium	30	75
Not concentrated conditioned medium	30	70

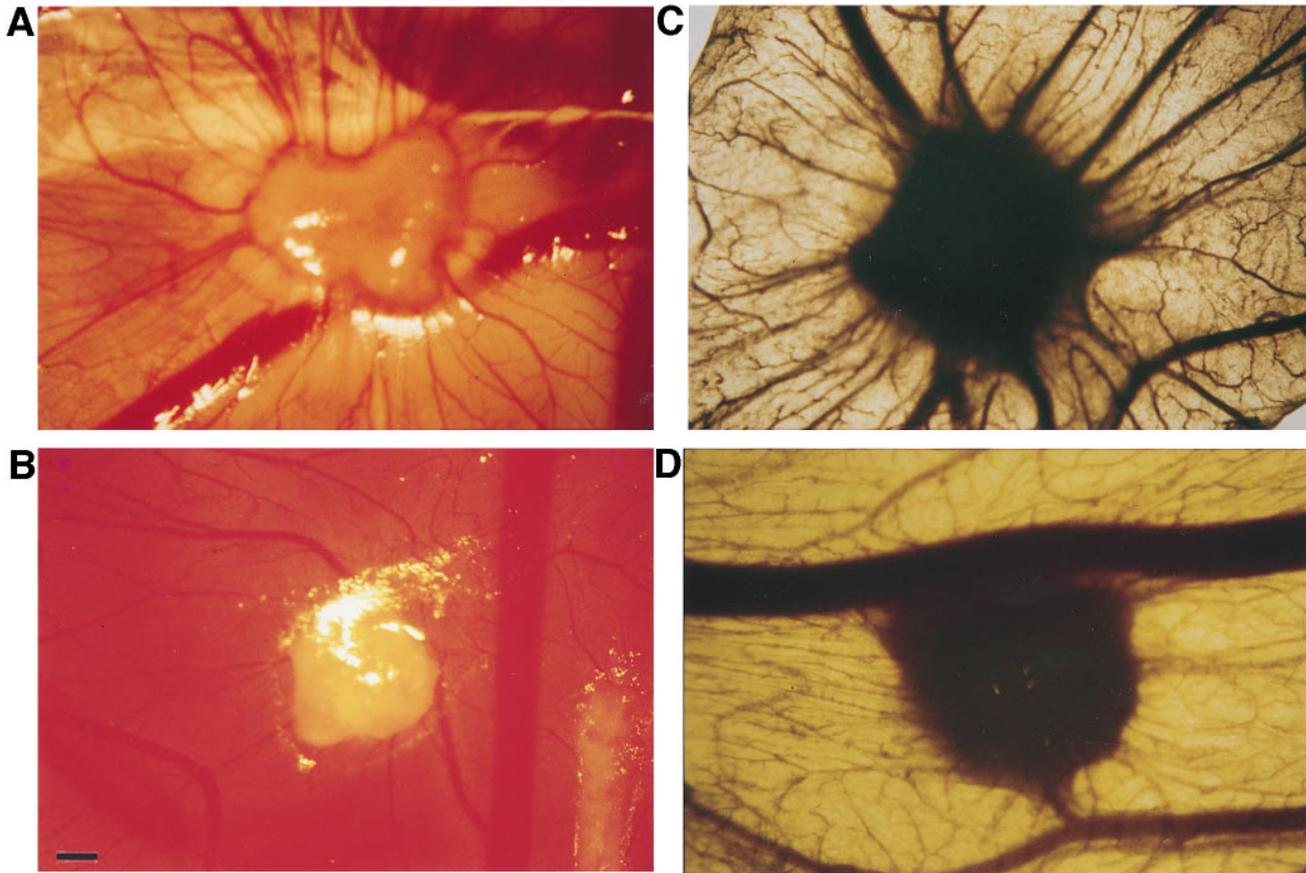


Figure 8. (A) CAM of 12-d-old chick embryo incubated for 4 d with a sponge adsorbed with 1 μg of ovotransferrin purified from chicken hypertrophic chondrocyte-conditioned medium. Note the presence of an increased number of blood vessels with a radially arranged "spoked wheel" pattern around the implant. (C) Vascular reaction is more detectable after India ink injection. (B and D) CAM of 12-d-old chick embryo incubated for 4 d with a sponge adsorbed with vehicle alone (PBS), used as negative control. No vascular response is detectable around the sponge in vivo (B) and after India ink injection (D). Bar, 330 μm .

After 12 d of incubation, macroscopically, the majority of the sponges were surrounded by allantoic vessels (Table III); vessels were more numerous in the sponges treated with purified ovotransferrin and developed radially toward the implant in a "spoked wheel" pattern (Fig. 8, A and C). In the specimens treated with PBS, no vascular reaction was detectable around the sponges (Fig. 8, B and D).

At a microscopic level, histological examination of the sponges showed a collagenous matrix containing numerous small blood vessels and fibroblasts localized between the sponge trabeculae (Fig. 9 a) 4 d after implantation. At the boundary between the sponge and the CAM mesenchyme, numerous host capillaries, penetrating in some points the sponge, were recognizable. In contrast, no collagenous matrix, blood vessels, or fibroblasts were present between the sponge trabeculae in the samples treated with PBS (Fig. 9 b).

Table IV shows quantitation of the angiogenic response performed at day 12 of incubation by using the planimetric point-count method (see Materials and Methods). In the experimental series, a higher microvessel density was detectable in the sponges treated with purified ovotransferrin, followed by sponges treated with concentrated conditioned medium, conditioned medium, ovotransferrin from chicken egg white, and apotransferrin from human plasma.

Discussion

Angiogenesis is a complex process characterized by a cascade of events including activation and migration of endothelial cells, degradation and remodeling of basement membrane and surrounding extracellular matrix, endothelial cell proliferation, and neovessel formation (Brown and Weiss, 1988; Folkman, 1984; Folkman and Shing, 1992). The process is activated by the synthesis and release of angiogenic factors and/or switching off of antiangiogenic factors by the cells.

It is generally believed that cartilage chondrocytes produce migration and growth inhibitors for endothelial cells able to prevent blood vessel invasion even in the presence of angiogenic stimulatory factors. Angiogenic inhibitors have been purified from both resting and proliferative cartilage (Moses et al., 1990, 1992; Pepper et al., 1991). A suppressed synthesis of these antiangiogenic factors together with an activated synthesis of angiogenic factors in hypertrophic chondrocytes could explain the invasion of hypertrophic cartilage by blood vessels. An angiogenic activity has been described to be associated with mineralized hypertrophic chondrocytes in vitro (Brown and McFarland, 1992; Descalzi Cancedda et al., 1995).

We have demonstrated the high levels of ovotransferrin

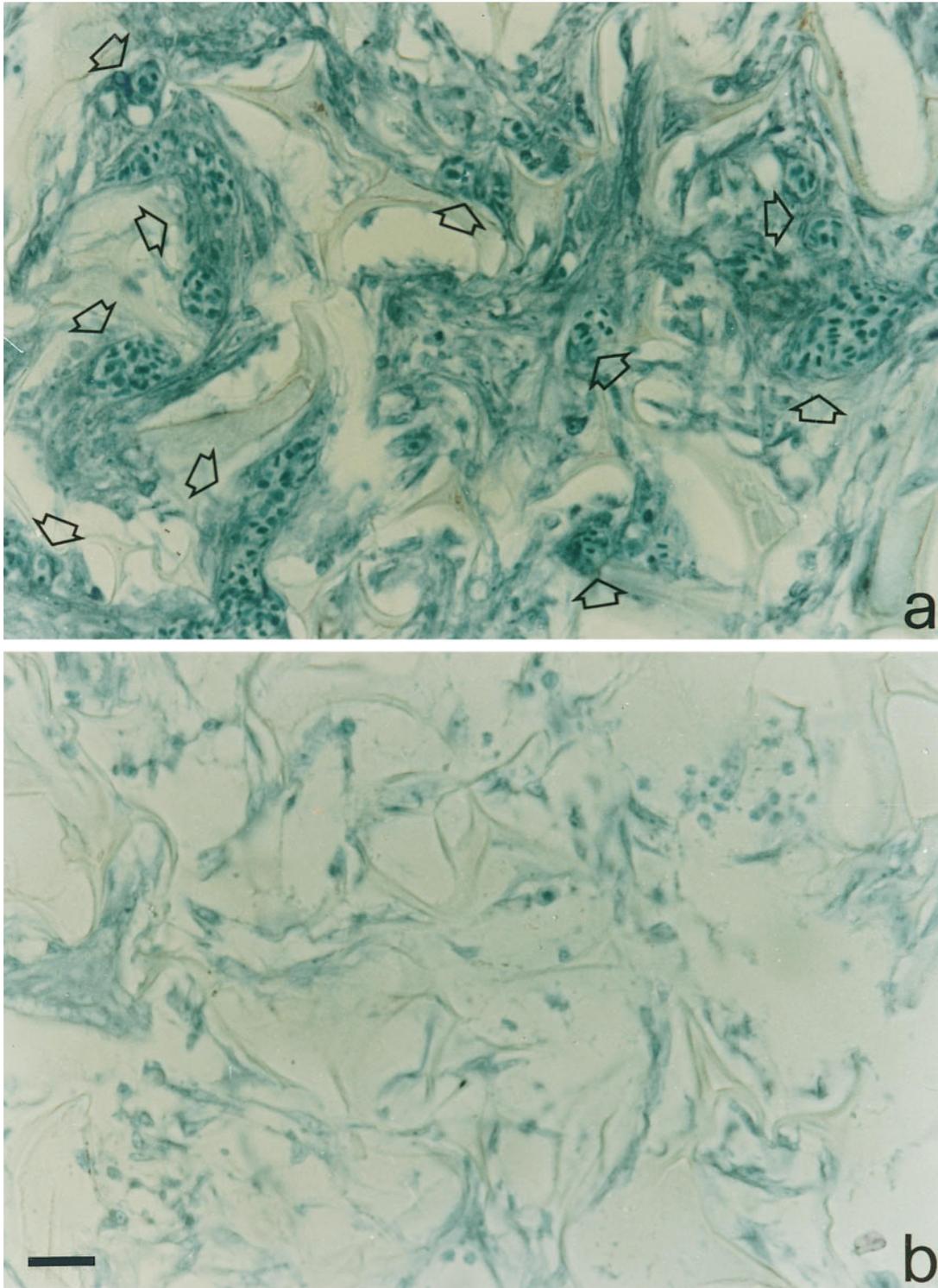


Figure 9. (a) Histological section of a sponge treated with purified ovotransferrin. Note, among the sponge trabeculae, a collagenous matrix containing numerous capillaries (*arrows*) and a cellular infiltrate prevalently constituted by fibroblasts. (b) Histological section of a sponge treated with PBS. No collagenous matrix, blood vessels, and fibroblasts are detectable among the sponge trabeculae. Bar, 50 μ m.

synthesis by hypertrophic chondrocytes both in vitro and in vivo (Gentili et al., 1993, 1994). Transferrins are monomeric, glycosylated, 80-kD proteins reversibly binding iron (de Jong et al., 1990). Hepatocytes are the main producer cells of this factor, but others such as Sertoli (Skinner et al.,

1989), oviduct (McKnight et al., 1980), ependymal (Tsumi et al., 1989), and oligodendroglial (Bloch et al., 1985) cells synthesize significant amounts of transferrin.

In addition, transferrin differentially stimulates the growth of metastatic variant lines of murine melanoma (Nicolson

Table IV. Quantitation of Angiogenic Response at Day 12 of Incubation

Treatment	Number	Number of	Microvessel
		intersection points (mean \pm SD)	
PBS	20	0	0
bFGF	20	30 \pm 2.4	22.2
Purified ovotransferrin	30	35 \pm 5.1	24.3
Chicken ovotransferrin	30	22 \pm 4.4	15.3*
Human apotransferrin	30	18 \pm 2.6	12.5*
Concentrated conditioned medium	30	30 \pm 7.2	20.8 [‡]
Not concentrated conditioned medium	30	25 \pm 4.8	17.4*

* $P < 0.001$ vs purified ovotransferrin.

[‡] $P < 0.01$ vs purified ovotransferrin.

et al., 1990) and human breast cancer cell lines (Inoue et al., 1993). Transferrin binding, but not growth response, correlates with invasive and metastatic properties of cell lines. This suggests that the transferrin/receptor system may be involved in more than just the support of cell proliferation. Endothelial cells have transferrin receptors (Jefferies et al., 1984) that are involved in transcytosis (Bickel et al., 1994); analogous to other cell types, these receptors could also be involved in endothelial cell proliferation and migration.

In the present study we have shown that transferrin is a chemotactic factor promoting migration and basement membrane invasion by endothelial cells. In the assay used to demonstrate this activity, endothelial cell migration was abolished by polyclonal antibodies directed against transferrin or by blocking antibodies directed against the transferrin receptor. We have previously shown a strong chemotactic and chemoinvasive activity, measured in Boyden chamber assays, in culture medium conditioned by hypertrophic chondrocytes (Descalzi Cancedda et al., 1995). In the presence of the antibodies against transferrin or the transferrin receptor, this activity decreased to 30–50% of the control. This finding indicates that transferrin is a major, but not the only, factor promoting cell migration in hypertrophic chondrocyte-conditioned culture medium and presumably in hypertrophic cartilage in vivo. Taking advantage of a serum-free culture system developed in our laboratory, we have shown that the medium conditioned by hypertrophic chondrocytes cultured in conditions in which they are surrounded by an organized extracellular matrix but do not express transferrin contained only 30% of the chemotactic activity normally observed in the media conditioned by hypertrophic chondrocytes. As expected, this residual activity was not affected by the addition of antibodies directed against the transferrin. From our study it appears that native transferrin in conditioned medium has a higher activity than purified transferrin. A possible synergism between transferrin and other factors present in the medium could explain this finding. An alternative explanation would be the possibility of partial inactivation of transferrin during purification.

In vivo studies on embryonic chorioallantoic membranes showed an angiogenic response to purified transferrins and conditioned culture medium. The angiogenic response of transferrin purified from chondrocyte culture medium and of concentrated conditioned culture medium

was comparable to that obtained with bFGF, a well-known angiogenic molecule.

We have shown a role for transferrin in promoting endothelial cell chemotaxis and chemoinvasion in vitro and angiogenesis in an in vivo assay. In evaluating the possible involvement of transferrin in promoting angiogenesis during tissue development, one should consider that the angiogenic process is the result of a balance between angiogenic and antiangiogenic factors. A high expression of transferrin in a given tissue, e.g., the liver, does not necessarily imply implementation of a neoangiogenic program, especially if antiangiogenic or other regulatory factors are also expressed. Transferrin appears to have a unique angiogenic activity, as, unlike other angiogenic factors, it did not promote proliferation in the endothelial cell line we have used for our studies.

Studies to determine the mechanisms of action of transferrin as a chemotactic agent and to identify additional factors acting with transferrin are currently under investigation. It is expected that these studies not only will allow a better understanding of the mechanisms controlling blood vessel invasion in the hypertrophic cartilage during endochondral bone formation, but also will contribute to an understanding of the relationship between control of cell proliferation and angiogenesis in other tissues including metastatic tumors.

We thank Dr. I.S. Trowbridge (Department of Cancer Biology, Salk Institute, San Diego, CA) for blocking mAbs against human transferrin receptor; Prof. M. Presta (University of Brescia, Italy) for kindly providing basic FGF; Mrs. Barbara Minuto for editorial help; and Dr. Douglas Noonan for revision.

This work was supported by a grant from Progetti Finalizzati "Applicazioni cliniche della ricerca oncologica," Consiglio Nazionale delle Ricerche, Rome, and by funds from the Associazione Italiana per la Ricerca sul Cancro and from Telethon, Italy.

Received for publication 11 April 1996 and in revised form 19 December 1996.

References

- Aizenman, Y., M.E. Wechsel, and J. de Vellis. 1986. Changes in insulin and transferrin requirements of pure brain neuronal cultures during embryonic development. *Proc. Natl. Acad. Sci. USA.* 83:2263–2266.
- Albini, A., Y. Iwamoto, H.K. Kleinman, G.R. Martin, S.A. Aaronson, J.M. Kozlowski, and R.N. McEwan. 1987. A rapid in vitro assay for quantitating the invasive potential of tumor cells. *Cancer Res.* 47:3239–3245.
- Albini, A., L. Repetto, S. Carlone, R. Benelli, R. Gendelman, P.D. Filippi, F. Bussolino, L. Monaco, M. Soria, and C. Parravicini. 1992. Characterization of Kaposi's sarcoma-derived cell cultures from an epidemic and a classic case. *Int. J. Oncol.* 1:723–730.
- Albini, A., G. Fontanini, L. Masiello, C. Tacchetti, D. Bigini, P. Luzzi, D.M. Noonan, and G. Stetler-Stevenson. 1994. Angiogenic potential in vivo by Kaposi's sarcoma cell-free supernatants and HIV-1 tat product: inhibition of KS-like lesions by tissue inhibitor of metalloproteinase-2. *AIDS (Phila.)* 8: 1237–1244.
- Albini, A., R. Benelli, M. Presta, M. Rusnati, M. Ziche, A. Rubartelli, G. Paglialunga, F. Bussolino, and D. Noonan. 1996. HIV-tat protein is a heparin-binding angiogenic growth factor. *Oncogene.* 12:289–297.
- Bickel, U., Y.S. Kang, T. Yoshikawa, and W.M. Pardridge. 1994. In vivo demonstration of subcellular localization of anti-transferrin receptor monoclonal antibody-colloidal gold conjugate in brain capillary endothelium. *J. Histochem. Cytochem.* 42:1493–1497.
- Bloch, B., T. Popovici, M.J. Levin, D. Tuil, and A. Kahn. 1985. Transferrin gene expression visualized in oligodendrocytes of the rat brain by using in situ hybridization and immunohistochemistry. *Proc. Natl. Acad. Sci. USA.* 82: 6706–6710.
- Brown, R.A., and C.D. McFarland. 1992. Regulation of growth plate cartilage degradation in vitro: effects of calcification and a low molecular weight angiogenic factor (ESAF). *Bone.* 17:49–57.
- Brown, R.A., and J.B. Weiss. 1988. Neovascularization and its role in the osteoarthritic process. *Annu. Rheum. Dis.* 47:881–886.

- Carmichael, J., W.G. Degraff, A.F. Gazdar, J.D. Minna, and J.B. Mitchell. 1987. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Res.* 47:936-942.
- Castagnola, P., G. Moro, F. Descalzi-Cancedda, and R. Cancedda. 1986. Type X collagen synthesis during in vitro development of chick embryo chondrocytes. *J. Cell. Biol.* 102:2310-2317.
- Cavanaugh, P.G., and G.L. Nicolson. 1991. Lung-derived growth factor that stimulates the growth of lung metastasizing tumor cells: identification as a transferrin. *J. Cell. Biochem.* 47:261-271.
- de Jong, G., J.P. van Dijk, and H.G. van Heijk. 1990. The biology of transferrin. *Clinica Chim. Acta.* 190:1-46.
- Descalzi Cancedda, F., C. Gentili, P. Manduca, and R. Cancedda. 1992. Hypertrophic chondrocytes undergo further differentiation in culture. *J. Cell. Biol.* 117:427-435.
- Descalzi Cancedda, F., A. Melchiori, R. Benelli, C. Gentili, L. Masiello, G. Campanile, R. Cancedda, and A. Albini. 1995. Production of angiogenesis inhibitors and stimulators is modulated by cultured growth plate chondrocytes during in vitro differentiation: dependence on extracellular matrix assembly. *Eur. J. Cell Biol.* 66:60-68.
- Edgell, C.J.S., C.C. McDonald, and J.B. Graham. 1983. Permanent cell line expressing human factor VIII-related antigen established by hybridization. *Proc. Natl. Acad. Sci. USA.* 80:3734-3737.
- Eklom, P., I. Thesleff, L. Saxen, A. Miettinen, and R. Timpl. 1983. Transferrin as a fetal growth factor: acquisition of responsiveness related to embryonic induction. *Proc. Natl. Acad. Sci. USA.* 80:2651-2655.
- Elias, H., and D.M. Hyde, editors. 1983. Stereological measurements of isotropic structures. In *A Guide to Practical Stereology*. Karger, Basel, Switzerland. 25-44.
- Folkman, J. 1984. What is the role of endothelial cells in angiogenesis? *Lab. Invest.* 51:601-604.
- Folkman, J. 1995. Angiogenesis in cancer, vascular rheumatoid arthritis and other diseases. *Na. Med.* 1:27-31.
- Folkman, J., and Y. Shing. 1992. Angiogenesis. *J. Biol. Chem.* 267:10931-10934.
- Gentili, C., P. Bianco, M. Neri, M. Malpeli, G. Campanile, P. Castagnola, R. Cancedda, and F. Descalzi-Cancedda. 1993. Cell proliferation, extracellular matrix mineralization, and ovotransferrin transient expression during in vitro differentiation of chick hypertrophic chondrocytes into osteoblast-like cells. *J. Cell Biol.* 122:703-712.
- Gentili, C., R. Doliana, P. Bet, G. Campanile, A. Colombatti, F. Descalzi-Cancedda, and R. Cancedda. 1994. Ovotransferrin and ovotransferrin receptor expression during chondrogenesis and endochondral bone formation in developing chick embryo. *J. Cell Biol.* 124:579-588.
- Inoue, T., P.G. Cavanaugh, P.A. Steck, N. Brünner, and G.L. Nicolson. 1993. Differences in transferrin response and numbers of transferrin receptors in rat and human mammary carcinoma lines of different metastatic potential. *J. Cell. Physiol.* 156:212-217.
- Jefferies, W.A., M.R. Brandon, S.V. Hunt, A.F. Williams, K.C. Gatter, and D.Y. Mason. 1984. Transferrin receptor on endothelium of brain capillaries. *Nature (Lond.)*. 312:162-163.
- Kleinman, H.K., M.L. McGarvey, J.R. Hassel, V.L. Star, F. Cannon, G.W. Laurie, and G.R. Martin. 1986. Basement membrane complexes with biological activity. *Biochemistry.* 25:312-318.
- Li, I., I. Kimira, and E.A. Ozawe. 1982. A myotrophic protein from chick embryo extracts: its purification, identity to transferrin and indispensability of avian myogenesis. *Dev. Growth Differ.* 94:366-377.
- McKnight, G.S., D.C. Lee, D. Hemmaphardh, C.A. Finch, and R.D. Palmiter. 1980. Transferrin gene expression. Effects of nutritional iron deficiency. *J. Biol. Chem.* 255:144-147.
- Menter, D.G., J.L. Herrmann, and G.L. Nicolson. 1995. The role of trophic factors and autocrine/paracrine growth factors in brain metastasis. *Clin. Exp. Metastasis.* 13(2):67-88.
- Moses, M.A., J. Sudhalter, and R. Langer. 1990. Identification of an inhibitor of neovascularization from cartilage. *Science (Wash. DC)*. 243:1408-1410.
- Moses, M.A., J. Sudhalter, and R. Langer. 1992. Isolation and characterization of an inhibitor of neovascularization from scapular chondrocytes. *J. Cell. Biol.* 119:475-482.
- Nicolson, G.L., T. Inoue, C.S. Van Pelt, and P.G. Cavanaugh. 1990. Differential expression of a $M_r \sim 90,000$ cell surface transferrin receptor-related glycoprotein on murine B16 metastatic melanoma sublines selected for enhanced brain and ovary colonization. *Cancer Res.* 50:515-520.
- Olivo, M., R. Bhardway, K. Schulze-Osthoff, C. Sorg, H. Jurgen-Jacob, and I. Flamme. 1992. A comparative study on the effects of tumor necrosis factor- α (TNF- α), human angiogenic factor (h-AF) and basic fibroblast growth factor (bFGF) on the chorioallantoic membrane of the chick embryo. *Anat. Rec.* 234:105-115.
- Partanen, G.L., I. Thesleff, and P. Eklom. 1984. Transferrin is required for early tooth morphogenesis. *Differentiation.* 27:59-66.
- Pepper, M.S., R. Montesano, J.D. Vassalli, and L. Orci. 1991. Chondrocytes inhibit endothelial sprout formation in vitro: evidence for involvement of a transforming growth factor-beta. *J. Cell. Physiol.* 146:170-179.
- Quarto, R., G. Campanile, R. Cancedda, and B. Dozin. 1992. Thyroid hormone, insulin, and glucocorticoids are sufficient to support chondrocyte differentiation to hypertrophy: a serum-free analysis. *J. Cell. Biol.* 119:989-995.
- Ribatti, D., C. Urbinati, B. Nico, M. Rusnati, L. Roncali, and M. Presta. 1995. Endogenous basic fibroblast growth factor is implicated in the vascularization of the chick embryo chorioallantoic membrane. *Dev. Biol.* 170:39-49.
- Salahuddin, S.Z., S. Nakamura, P. Biberfeld, M.H. Kaplan, P.D. Markham, L. Larsson, and R.C. Gallo. 1988. Angiogenic properties of Kaposi's sarcoma-derived cells after long-term culture in vitro. *Science (Wash. DC)*. 242:430-432.
- Skinner, M.K., S.M. Schlitz, and C.T. Anthony. 1989. Regulation of Sertoli cell differentiated function: testicular transferrin and androgen binding protein expression. *Endocrinology.* 124:3015-3024.
- Thompson, E., S. Nakamura, T. Shima, A. Melchiori, G. Martin, S. Salahuddin, R. Gallo, and A. Albini. 1991. Supernatants of acquired immunodeficiency syndrome-related Kaposi's sarcoma cells induce endothelial cell chemotaxis and invasiveness. *Cancer Res.* 51:2670-2676.
- Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 78:3039-3043.
- Trowbridge, I.S., and F. Lopez. 1982. Monoclonal antibody to transferrin receptor blocks transferrin binding and inhibits human tumor cell growth in vitro. *Proc. Natl. Acad. Sci. USA.* 79:1175-1179.
- Trowbridge, I.S., and B. Omary. 1981. Human cell surface glycoprotein related to cell proliferation is the receptor for transferrin. *Proc. Natl. Acad. Sci. USA.* 78:3039-3043.
- Tsutsumi, M., M.K. Skinner, and E. Sanders-Bush. 1989. Transferrin gene expression and synthesis by cultured choroid plexus epithelial cells. *J. Biol. Chem.* 264:9626-9631.