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Tumor-induced endothelial cell activation: role of vascular endothelial growth factor

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Castilla, M. Ángeles, Fernando Neria, Guadalupe Renedo, Daniel S. Pereira, Francisco R. González-Pacheco, Sonsoles Jiménez, Paloma Tramón, J. J. P. Deudero, M. V. Alvarez Arroyo, Susana Yagüe, and Carlos Caramelo. Tumor-induced endothelial cell activation: role of vascular endothelial growth factor. *Am J Physiol Cell Physiol* 286: C1170–C1176, 2004. First published January 7, 2004; 10.1152/ajpcell.00306.2003.—Proangiogenic, proliferative effects of tumors have been extensively characterized in subconfluent endothelial cells (EC), but results in confluent, contact-inhibited EC are critically lacking. The present study examined the effect of tumor-conditioned medium (CM) of the malignant osteoblastic cell line MG63 on monolayer, quiescent bovine aorta EC. MG63-CM and MG63-CM + $CoCl_2$ significantly increased EC survival in serum-starved conditions, without inducing EC proliferation. Furthermore, MG63-CM and MG63-CM + $CoCl_2$, both containing high amounts of vascular endothelial growth factor (VEGF), induced relevant phenotypic changes in EC (all $P < 0.01$) involving increase of nucleoli/chromatin condensations, nucleus-to-cytosol ratio, capillary-like vacuolated structures, vessel-like acellular areas, migration through Matrigel, growth advantage in reseeding, and factor VIII content. All these actions were significantly inhibited by VEGF and VEGF receptor (VEGFR2) blockade. Of particular importance, a set of similar effects were detected in a human microvascular endothelial cell line (HMEC). With regard to gene expression, incubation with MG63-CM abolished endogenous VEGF mRNA and protein but induced a clear-cut increase in VEGFR2 mRNA expression in EC. In terms of mechanism, MG63-CM activates protein kinase B (PKB)/Akt, p44/p42-mitogen-activated protein kinase (MAPK)-mediated pathways, as suggested by both inhibition and phosphorylation experiments. In conclusion, tumor cells activate confluent, quiescent EC, promoting survival, phenotypic, and gene expression changes. Of importance, VEGF antagonism converts MG63-CM from protective to EC-damaging effects.

vascular endothelial growth factor receptor 2; MG63-conditioned medium

ENHANCED ANGIOGENESIS is a major contributor to the poor clinical outcome of tumors. However, the mechanisms involved in tumor-related angiogenesis are still incompletely understood. In the past half-century, numerous studies have dealt with the effects of tumor cells and tumor-conditioned media (CM) on endothelial cells (EC). Mainly, these studies have demonstrated that malignant cells produce a host of factors that induce EC growth and favor vascular permeability, therefore facilitating tumor spreading (14, 15, 22). In other words, in the particular biology of the tumors, EC encompass the growth of malignant cells by means of a growth factor signaling network. However, data are lacking on the effects of tumors on quiescent EC organized in a confluent, monolayer distribution, as found in normal vessels. These effects are particularly important, because they involve the type of interaction that tumors establish in vivo with intact endothelia, e.g., in the case of metastatic seeding.

Although vascular endothelial growth factor (VEGF) is a *primum inter pares* among all the agents involved in tumor vascularization, its specific role still has several major unknown aspects (12, 13). In a general view, VEGF and its tyrosine kinase receptors VEGFR1 (flt-1) and VEGFR2 (flk-1/KDR) are key mediators of physiological and pathological angiogenesis. They are expressed in most tissues during embryonic development but tend to be downregulated in the adult (12, 13). VEGFR2 mediates increased EC mitogenesis and permeability, whereas the role of VEGFR1 has not been yet sufficiently elucidated.

Upregulation of VEGF and VEGFRs has been observed during the induction of angiogenesis (12, 13). Such upregulation is particularly intense in hypoxic and neoplastic tissues. In the latter, current opinion maintains that tumor cells produce VEGF and stimulate VEGFR2 expression, therefore closing a loop of EC activation (18, 22). Although in this setting the pattern of expression of VEGFR2 appears to parallel VEGF expression, little is known about their mutual interaction and regulation. Moreover, tumor-produced VEGF can theoretically influence EC-produced, autologous endothelial VEGF; no data are yet available, however, to substantiate this hypothesis. Collectively, compelling evidence suggests that VEGF and its receptors are critical for tumor-associated angiogenesis and that they represent good targets for therapeutic intervention (22); however, data are still lacking for full understanding of the complete mechanisms of action of anti-VEGF strategies. In this regard, no description is currently available of the precise tumor-induced changes in confluent EC and the role of VEGF in each of these changes.

In light of the aforementioned rationale, the aim of the present study was to analyze the effect of tumors on monolayer, quiescent EC, with a focus on the role of VEGF and its receptors.

MATERIALS AND METHODS

EC culture. Bovine aorta EC were obtained, characterized, and cultured as described previously (7, 19, 20) in accordance with the

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Guiding Principles for Research Involving Animals and Human Beings" of the American Physiological Society. Furthermore, comparative experiments were done with a human microvascular endothelial cell line (HMEC). HMEC were a gift from Dr. Manuel Ortiz de Landazuri (Immunology Laboratory, Hospital de la Princesa, Madrid, Spain).

The conditions were modified when needed for the different experiments. Unless stated otherwise, all the experiments were done on confluent EC. Anti-VEGF (Sigma, St. Louis, MO) and anti-VEGFR2 (3.83; Imclone, New York) MAbs were used for blockade of VEGF actions. Both antibodies have been extensively tested for their blocking properties in our laboratory and by the manufacturers (1, 7, 8). Because of its scarce availability, the noncommercial MAb 3.83 was only used in selected experiments. Crystal violet, hematoxylin and eosin, Masson trichrome, and immunocytochemistry techniques were applied to the EC in different conditions. Both the number of nucleoli/chromatin condensations and the nucleus-to-cytosol diameter ratio were calculated on digitized microphotographs examined by three independent observers who were blinded for the experimental conditions.

MG63 culture and CM preparation. Human MG63, a line of malignant osteoblast-like cells (American Type Culture Collection), were maintained in Dulbecco's modified Eagle's medium (DMEM) + 10% FBS. For preparing CM, MG63 were cultured in MEM-D-valine without FBS for 24 h; medium was collected, filtered, and added to the EC without further maneuvers. When needed, a similar incubation was carried out with the hypoxia-mimicking agent $CoCl_2$ (10 μM) added to the medium.

EC damage and proliferation. EC damage was addressed by flow cytometry (7). Lactate dehydrogenase (LDH) release was assessed in confluent EC maintained for 24–48 h in growth factor-deficient conditions (MEM-D-valine without FBS) (7, 8). Two hundred microliters were sampled at different times, and percent LDH release was calculated. Cell counting was performed in a Neubauer chamber. Cell proliferation was quantified with a cell proliferation ELISA bromodeoxyuridine (BrdU) kit (Roche, Madrid, Spain). Experiments with a Transwell system were used for assessing the direct effect of the presence of MG63 on EC (see Fig. 1*C* for description).

mRNA isolation and reverse transcriptase-PCR. Total RNA was extracted as described previously with the TriPure isolation reagent (Boehringer Mannheim, Madrid, Spain) after different times of exposure (3, 9, 24 h) to the treatments (see below for further details) (1, 7). Reverse transcriptase (RT)-PCR was performed with oligonucleotide primers of bovine VEGF, VEGFR1, and VEGFR2, synthesized as described previously (7).

VEGF Western blot and ELISA. Samples were processed and analyzed by Western blotting as described previously (7). Anti-VEGF MAb (1:250; Sigma) was used as antibody. VEGF concentrations in MG63-CM were measured by a VEGF ELISA (Accucyte Human VEGF, Cytimmune Sciences).

p44/p42-mitogen-activated protein kinase and Akt Western blots. Western blots for phospho-p44/p42-mitogen-activated protein kinase (MAPK), phospho-Akt (Ser473), and their respective loading controls [total p44/p42-MAPK and protein kinase B (PKB)/Akt] were performed following the manufacturer's instructions (Cell Signaling Technology, Beverly, MA). The phosphatidylinositol 3-kinase (PI3-kinase) inhibitor LY-294002 (20 μM; Sigma) and the MAPK kinase (MEK) inhibitor PD-98059 (50 μM; Calbiochem, La Jolla, CA) were used during the 45 min before addition of the medium.

Matrigel invasion assay. The activated state of EC was further assessed by their penetration into growth factor-reduced Matrigel (Becton Dickinson, Barcelona, Spain).

Immunocytochemistry of factor VIII expression. Cells grown to confluence on tissue culture chamber slides (Becton Dickinson USA) were submitted to the different experimental maneuvers, washed with PBS, incubated in MEM-D-valine-0.5% FBS, MG63-CM, and MG63-CM-$CoCl_2$ for an additional 5 days, and then fixed with Merckofix (Merck). Immunostaining was performed by the alkaline phosphatase method, using factor VIII (1:1,000, polyclonal rabbit; Sigma) as primary antibody. Similarly fixed cultures were treated with nonimmunogenic rabbit IgG as negative control. After being washed with PBS, cells were incubated with biotinylated swine anti-rabbit IgG (Dako, Glostrup, Denmark) for 30 min, sequentially followed by incubation with avidin-biotin-peroxidase complex (Dako) and 3,3′-diaminobenzidine (Sigma).

Statistics. Results are expressed as means ± SE. Unless stated otherwise, each value corresponds to a minimum of five triplicate experiments. Comparisons were done by ANOVA or paired and unpaired Student's *t*-test, when appropriate. The Fisher and Scheffé tests for multiple comparisons were used to determine the *P* value, which was considered significant at <0.05. All statistic analyses were performed with the SPSS 8.0 package (Jandel, San Rafael, CA).

RESULTS

Tumor-CM-induced changes in confluent, quiescent EC. Exposure to MG63-CM significantly increased survival of EC in serum-free conditions, as assessed by flow cytometry (Fig. 1*A*). A similar protective effect by MG63-CM was obtained on the microvascular endothelial line HMEC (Fig. 1*B*). EC-CM had no effect on EC survival. In the same regard, MG63-CM significantly decreased LDH release [fresh media (FM), 100 ± 3.9%; 20% FBS, 50 ± 3.4% ($P < 0.001$ with respect to FM); MG63-CM, 54 ± 4.2% ($P < 0.001$ with respect to FM); CM of EC, 98 ± 3.8%]. A related effect was obtained by coincubating EC with MG63 in a Transwell system (Fig. 1*C*).

In addition, we found that, in the absence of FBS, no cell proliferation, as assessed by percent BrdU incorporation, occurred in confluent, quiescent EC treated with MG63-CM or exogenous VEGF (FM: 68 ± 3%, MG63-CM: 66 ± 9%, MG63-CM + $CoCl_2$: 53.8 ± 7%, 5×10^{-10} M VEGF: 68.8 ± 4%; $n = 3$ triplicate experiments). In these conditions, 20% FBS induced an increase in BrdU incorporation of 217 ± 43% ($P < 0.001$). Furthermore, no changes in either glucose or pH of the different media were detected after 24-h incubation (data not shown). Of importance, MG63-CM contained increased amounts of VEGF (FM: undetectable levels, MG63-CM: 13.5 ± 1.2 ng/ml; $P < 0.01$); VEGF levels were even higher in the presence of $CoCl_2$ (MG63-CM + 10 μM $CoCl_2$: 26.8 ± 1.5 ng/ml; $P < 0.01$ with respect to MG63-CM alone and $P < 0.001$ with respect to FM).

Incubation with MG63-CM induced relevant phenotypic changes in confluent EC, which were consistent with an activated state. Of specific importance, no phenotypic changes were detected by treating the EC with exogenous VEGF (5×10^{-10} M) or with EC-CM (with or without $CoCl_2$; images not shown). The changes produced by MG63-CM are depicted in Fig. 2 and include the following. *1*) The number of nucleoli/chromatin condensations (EC + MG63-CM 5.3 ± 0.7 vs. EC-FM 3.4 ± 0.4 nucleoli/cell; $P < 0.01$) and the nucleus-to-cytosol ratio (FM 0.41 ± 0.09 control; MG63-CM-$CoCl_2$ 0.68 ± 0.08; $P < 0.01$) were increased. *2*) Acellular areas appeared, consistent with attempted capillarization (Fig. 2*B*,*f*). *3*) Vacuolated, seal-shaped EC (Fig. 2, *A*,*c* and *B*,*d*) appeared; these structures were also consistent with attempted capillarization in bidimensional growth conditions and resemble those found in malignant angiomas (17). The aforementioned changes were more marked when the EC were incubated with MG63-CM + $CoCl_2$ (10 μM). *4*) Migration of the EC within Matrigel occurred; no significant migration occurred by incu-

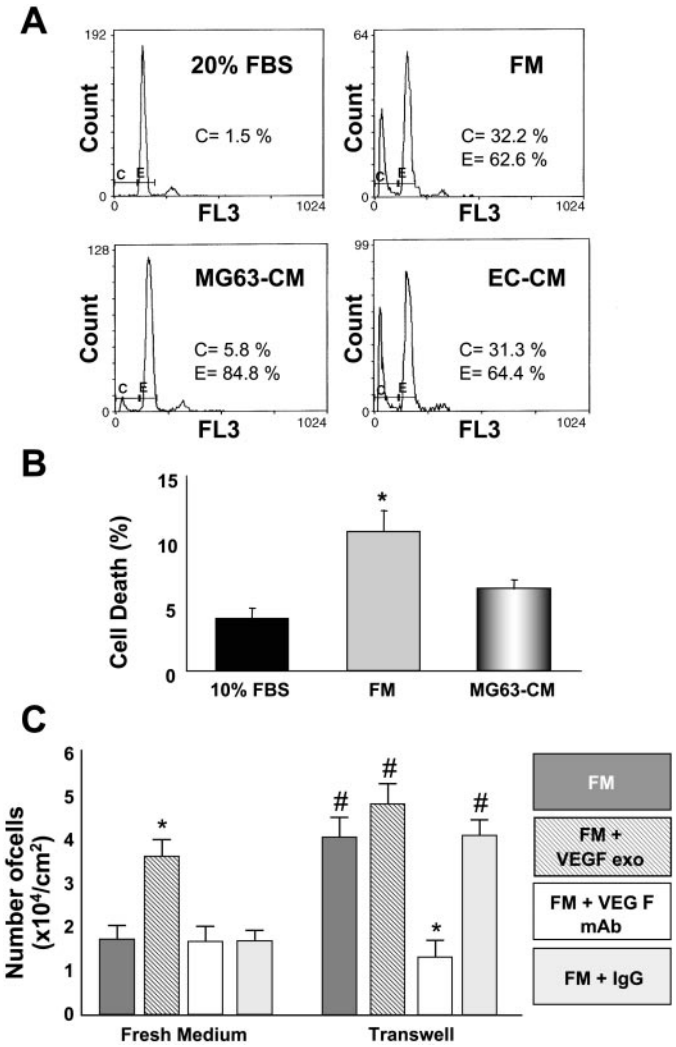

Fig. 1. *A*: flow cytometry analysis. Endothelial cells (EC) were analyzed for DNA content by propidium iodide staining and flow cytometry. Measurements were made 48 h after FBS deprivation (1%). Numbers show percentage of living (E) or dead (C) cells. *x*-Axis, fluorescence intensity of propidium iodide; *y*-axis, cell number. Traces are representative of a minimum of 5 experiments with similar results. *B*: cell death of human microvascular endothelial cells (HMEC) by flow cytometry analysis. The effects of 10% FBS, fresh medium (FM), and MG63-conditioned medium (CM) are shown. Bars represent the mean values of 4 triplicate experiments. Different concentrations of FBS were used in EC and HMEC (20% and 10%, respectively) because of the different growth rate of the two types of cells. $^{*}P < 0.01$ with respect to the other 2 conditions. *C*: Transwell experiments. EC were cultured to confluence in P6 plates and MG63 were seeded at 10^4 cells/cm^2 and incubated 24 h in Millicell-PCF. Both cell types were serum deprived in MEM-D-valine with 1% FBS 24 h before the coincubation and thereafter incubated together in MEM-D-valine in the presence of exogenous (exo) vascular endothelial growth factor (VEGF; 10 ng/ml), anti-VEGF MAb (1 μg/ml), or nonspecific IgG (1 μg/ml). Bars on *left* represent different controls in the absence of MG63; bars on *right* represent the coincubation experiment in Transwell ($n = 5$). $^{*}P < 0.01$ with respect to the other 3 columns of the group; $^{\#}P < 0.05$ with respect to fresh medium without MG63.

bating the EC with FM (Table 1). *5*) Treatment with MG63-CM and MG63-CM-$CoCl_2$ induced a marked increment in factor VIII, as assessed by immunocytochemistry. Moreover, factor VIII distribution was different in MG63-CM-treated EC with respect to nonconditioned media or EC-CM-treated EC (Fig. 2*C*). Changes induced by the addition of anti-VEGF antibody are commented on below. *6*) Pretreatment of EC with MG63-CM induced growth advantage in reseeding in FBS-poor conditions. This growth advantage was more marked when the CM was obtained from MG63 + $CoCl_2$ (Table 2).

Additional experiments were performed with HMEC to analyze whether the findings in EC originated in a great vessel were also valid in EC of microvascular type. HMEC growth rate and phenotype were rather different than those of EC (Fig. 2*D*); however, a significant protective effect on HMEC was also found in the presence of MG63-CM (Figs. 1*B* and 2*D*).

Role of VEGF and VEGFR2-mediated pathways in changes induced by MG63-CM on confluent, quiescent EC. After the aforementioned alterations were identified, we analyzed whether VEGF was a critical factor in the protective effect of MG63-CM. These experiments revealed a remarkably important fact, i.e., that the inhibition of VEGF effects by a specific anti-VEGF antibody not only blocks the protection induced by MG63-CM but also unmasks a deleterious effect of MG63-CM on EC (Fig. 3, *A* and *B*). In HMEC, however, both anti-VEGF and anti-VEGFR2 induced a complete reversal of the MG63-CM protective effect, but without inducing a significant increase in cell death over the baseline (Fig. 3*C*).

Treatment with the anti-VEGF antibody completely blocked the effect of MG63-CM on EC phenotype (Fig. 2) and migration within Matrigel (Table 1). In the same regard, the number of nucleoli, as well as the nucleus-to-cytosol ratio, were significantly reduced in the presence of anti-VEGF MAb (MG63-CM-$CoCl_2$ + anti-VEGF MAb: 3.9 ± 0.6 nucleoli/cell and nucleus-to-cytosol ratio 0.41 ± 0.06, both $P < 0.01$ with respect to MG63-CM-$CoCl_2$ without anti-VEGF MAb; for both measurements, see above for comparison). As shown in Fig. 2, acellular spaces were markedly reduced when the anti-VEGF MAb was present in the media. Moreover, the formation of vacuolated capillary-like structures was almost abolished in the presence of the anti-VEGF MAb (2 ring shaped/402 EC; $P < 0.001$; Fig. 2) compared with EC treated with MG63-$CoCl_2$ (98 ring shaped/394 EC) or MG63-$CoCl_2$ + nonspecific IgG (72 ring shaped/376 EC). In addition, treatment with anti-VEGF MAb significantly decreased the MG63-CM-induced increase in factor VIII expression; an identical image was observed by using anti-VEGFR2 (MAb 3.83). All the experiments using anti-VEGF or anti-VEGFR2 antibodies included controls incubated in identical conditions but exposed to nonspecific IgG of the same type of anti-VEGF and 3.83 antibodies. No changes were detected in these controls with respect to MG63-CM or FM without antibodies (data not shown).

Role of tumor-CM on autologous VEGF and VEGFR2 mRNA expression in EC. Incubation with MG63-CM induced major changes in the expression of VEGF by EC. MG63-CM markedly inhibited autologous VEGF mRNA compared with the expression detected in the presence of FM (Fig. 4*A*); a similar result was obtained for VEGF protein (Fig. 4*B*). The fact that the addition of anti-VEGF MAb was not capable of blocking the MG63-CM-induced abolition of autologous VEGF expression indicates that this abolition was not related to the presence of VEGF in the MG63-CM. Therefore, molecules different from VEGF contained in the MG63-CM are probably involved, e.g., other growth factors.

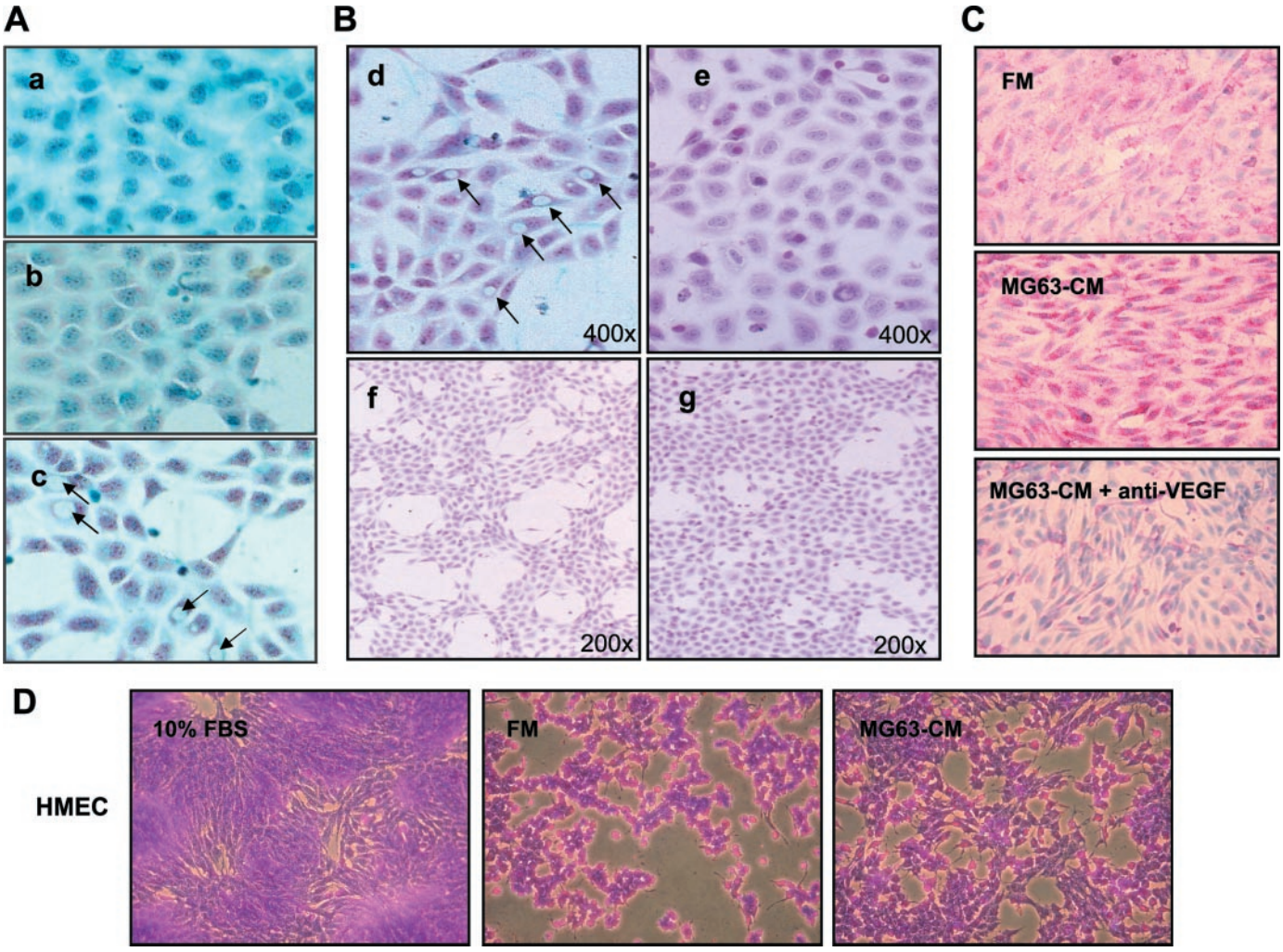

Fig. 2. Phenotypic changes after exposure to tumor cell (MG63)-CM and MG63-CM + $CoCl_2$. *A* and *B*: Masson stain. *C*: immunocytochemistry of factor VIII expression. *D*: crystal violet stain. Experiments were done both in the presence and the absence of anti-VEGF MAb. *A*: *a*: control EC (MEM-D-valine-1% FBS); *b*: EC treated with MG63-CM; *c*: EC treated with MG63-CM + $CoCl_2$. Compared with *a*, in *b* and *c* cell junctions are widened and acellular areas have appeared. Moreover, the nuclear size has increased in relation to the cytosol. Changes are more marked, and capillary-like, seal-shaped structures, are evident in *c* (arrows) (×400). *B*: *d* and *f*: EC exposed to MG63-CM + $CoCl_2$; *e* and *g*: EC exposed to MG63-CM + $CoCl_2$ + anti-VEGF MAb. As can be observed, the anti-VEGF MAb blocked almost completely the induction of the seallike capillary structures (arrows, *d*). On the other hand, only a partial regression of the acellular areas was observed (*g*). *C*: MG63-CM increased factor VIII in EC in a VEGF-dependent manner, as assessed by simultaneous treatment with anti-VEGF MAb. *D*: HMEC incubated for 48 h in 3 different conditions: 10% FBS, fresh medium (FM), and MG63-CM.

To further analyze the mechanisms involved in the effect of MG63-CM, the expression of VEGFR2 mRNA was assessed by RT-PCR. As can be seen in Fig. 5, MG63-CM induced an increase in VEGFR2 mRNA expression in samples studied at 0, 3, 9, and 24 h. In terms of mechanism, the VEGFR2 increase was critically related to a paracrine effect secondary to VEGF present in the MG63-CM. This VEGF acts through the VEGFR2 receptor, as judged by the marked decrease in the stimulation of VEGFR2 expression in the presence of anti-VEGF antibody and anti-VEGFR2 antibodies (Fig. 5). With respect to the VEGFR1/flt-1 receptor, no differences in mRNA expression were detected in the same samples used for the study of VEGFR2 expression (Fig. 5).

Effect of MG63-CM on Akt and MAPK phosphorylation. Additional experiments were performed to analyze the putative pathways involved in EC activation by MG63-CM. The pathways analyzed were chosen on basis of the known signaling routes of VEGF. Incubation of confluent EC in the presence of MG63-CM induced a significant increase in Akt and MAPK phosphorylation (Fig. 6). Both increased phosphorylations were blocked in the presence of the PI3-kinase inhibitor LY-294002 and the MEK inhibitor PD-98059, respectively (Fig. 6).

DISCUSSION

The data obtained in the present study are useful to clarify previously unknown aspects of the behavior of EC in the tumor microenvironment, referring not just to EC proliferation but to a spectrum of EC activation. More specifically, some of our findings apply to the effects on angiogenic events that are related to the occurrence of hypoxia within malignant tumors,

Table 1. *Effect of MG63-CM on Matrigel penetration by endothelial cells*

	EC/field
FM	12±5
FM+IgG	14±3
FM+VEGF MAb	17±6
CM	237±36*
CM+IgG	220±42*
CM+VEGF MAb	88±15*†
FM+3.83	15±5
CM+3.83	16±5‡

Values are means ± SE. Endothelial cells (EC) were tested for their ability to migrate in response to MG63-conditioned medium (CM). EC were seeded at 10^4 cell/well and incubated 48 h with the different media. EC/field, no. of cells that have traversed the Matrigel, as counted in marked fields. FM, fresh medium, no FBS. CM: MG63-CM, anti-VEGF MAb (1 μg/ml), 3.83 MAb (0.5 μg/ml), or IgG (0.5 μg/ml). *$P < 0.001$ with respect to FM. †$P < 0.01$ with respect to CM. ‡$P < 0.001$ with respect to CM.

as assessed by means of MG63-CM with $CoCl_2$. A specific feature of the present study is that the EC were in the confluent, monolayer state actually adopted by quiescent endothelia in the normal vessel wall. This issue is particularly relevant to understanding the response of the endothelium in metastatic seeding; in the latter case, tumor cells interact with a priori intact endothelia in different organs.

The literature on the effects of tumor-CM on EC was mostly generated in the 1970s and 1980s. The studies were mainly focused on two major subjects, namely, the effect of tumors on EC proliferation and tumor-induced endothelial permeability (6, 15). Rather unexpectedly, the aforementioned studies contained almost no information on the actions of tumor cells and/or tumor cell-CM on confluent EC in the contact-inhibited state.

Our study has detected significant effects of tumor-CM on EC phenotype, indicating activation. To our knowledge, the pattern of EC activation in the presence of tumor-CM and tumor cells, namely, MG63-CM, is described for the first time, and it includes marked increases in nuclear size and number of nucleoli/cromatin condensations, as well as formation of capillary-like structures. The changes found in our experiments with MG63-CM are particularly illustrative of the magnitude of the activation of normal EC in the tumor microenvironment and resemble those described in vivo as an angiogenic phenotype (21). The effects on factor VIII expression are of special interest regarding the potential role of VEGF in the procoagulating activity of tumors and deserve further attention.

Table 2. *EC growth advantage after pretreatment with MG63-CM*

	48 h	72 h
FM	4.6±0.8(46)	6.4±0.7(64)
FM+IgG	4.5±0.3(45)	6.2±1.1(62)
FM+VEGF MAb	5.5±0.7(48)	6.7±0.8(67)
CM	9.9±1.3(99)†	5.9±1.1(59)
CM+VEGF MAb	5.0±1.2(50)	3.6±1.0(36)†
CM+$CoCl_2$	10.6±0.8(106)†	8.4±1.5(84)†
CM+$CoCl_2$+VEGF MAb	5.0±1.1(50)	5.0±0.8(50)*

Values (expressed as 10^4 cells/cm^2) are means ± SE. Confluent EC were incubated 24 h with the different media; thereafter, EC were trypsinized and reseeded in MEM-D-valine with 1% FBS. Cells were counted at 48 and 72 h. FM, serum-deprived medium; CM, MG63-CM + $CoCl_2$ (10 μM); values in parentheses are % yield of the reseeding with respect to total EC seeded. *$P < 0.05$, †$P < 0.01$ with respect to FM.

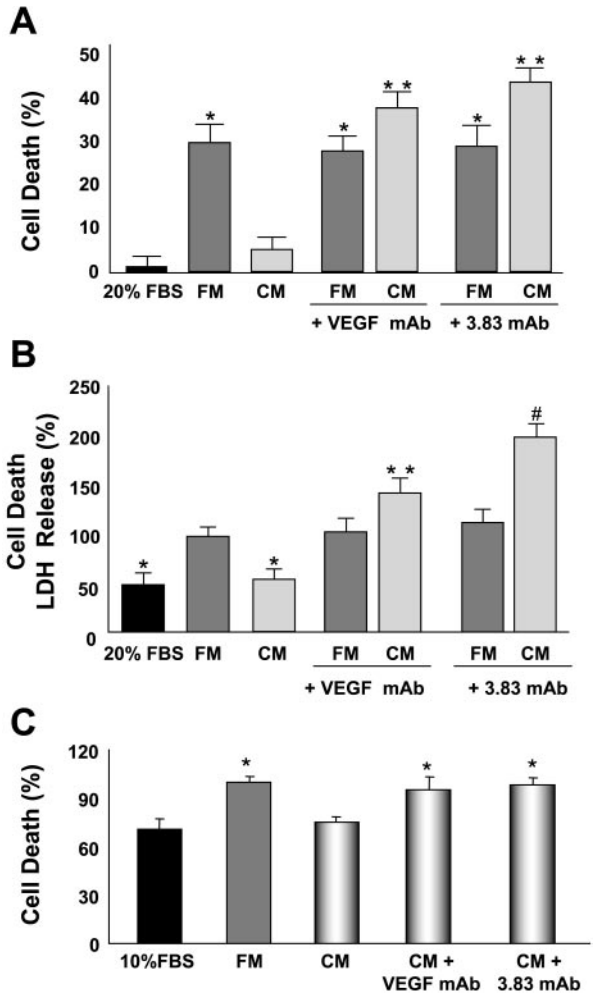

Fig. 3. Cytoprotective effect of MG63-CM on EC. All incubations, except those specifically identified, were done in the absence of FBS. Data are means ± SE of 4 experiments with triplicate samples. Anti-VEGF MAb: 1 μg/ml; 3.83 MAb [anti-VEGF receptor (VEGFR)2]: 0.5 μg/ml. *A*: % of cell death at 48 h (flow cytometry). *$P < 0.01$ with respect to 20% FBS; **$P < 0.01$ with respect to CM and $P < 0.05$ with respect to FM ($n = 4$; triplicate experiments). *B*: % of lactate dehydrogenase (LDH) release; 100% cell death was assigned to FM (fresh serum-deprived medium). *$P < 0.05$ decrease with respect to FM; **$P < 0.05$ increase with respect to FM; #$P < 0.01$ increase with respect to FM ($n = 4$; triplicate experiments). *C*: relative cell death measured by flow cytometry analysis at 48 h. Effects of the different incubation conditions on HMEC (100% cell death was assigned to FM) are shown. As can be seen, both anti-VEGF and anti-VEGFR2 antibodies inhibited the protective effect of MG63-CM. No effects of anti-VEGF and anti-VEGFR2 antibodies were detected in the incubations with fresh medium. *$P < 0.05$ with respect to CM.

In addition to the morphological changes, our data demonstrate that soluble factors present in MG63-CM have protective effects against FBS deprivation. Of utmost importance, for its significance in terms of putative therapeutic applications, this protective response of MG63-CM became deleterious when VEGF was blocked. This finding reveals, for the first time, that on blockade of the key protective mediator VEGF, EC damage is significantly potentiated by tumor-borne products and therefore highlights the importance of autologous VEGF.

The anti-EC effects of MG63-CM in the presence of VEGF or VEGFR2 blockade are most probably conveyed through

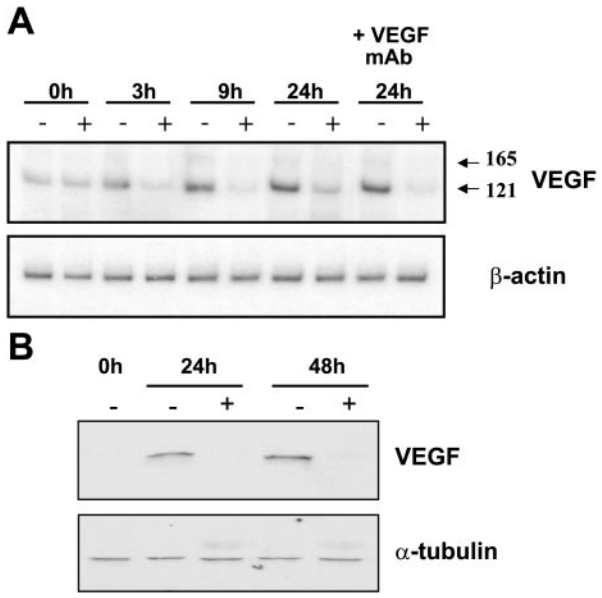

Fig. 4. A: blockade of expression of VEGF mRNA at different times as assessed by RT-PCR. The assays were carried out in the presence of MG63-CM or FM: +, CM; −, FM. Times indicate duration of incubation before RNA isolation. β-Actin was used as internal control. VEGF mRNA increased progressively in the serum-deprived conditions but not when MG63-CM was present. Images are representative of 4 experiments with similar results. *B*: inhibition of autocrine VEGF expression by MG63-CM. Images of VEGF Western blot in protein samples extracted from cells treated with MG63-CM (+) or FM (−) for 24 or 48 h or nontreated cells (0 h) are shown. α-Tubulin was used as control of equal gel loading. Images are representative of 3 experiments with similar results.

soluble factors. Several antiangiogenic agents have been described in tumors, e.g., angiostatin, endostatin, and thrombospondin (10). However, the precise identification of the deleterious factors contained in the MG63-CM, albeit of interest, is beyond the specific aims of the present research.

Our results also show that the effects of tumor-CM and VEGF blockade are relevant in EC of microvascular origin. The existence of some differences between the findings in EC and HMEC, e.g., death rate, phenotype, and time to confluence, can be traced to the fact that HMEC are a continuous cellular line with a markedly different growth rate and, more importantly, with metabolic modifications aimed to ensure a more efficient survival. In this regard, the results obtained in the EC in primary culture are probably closer to the actual properties of EC in vivo.

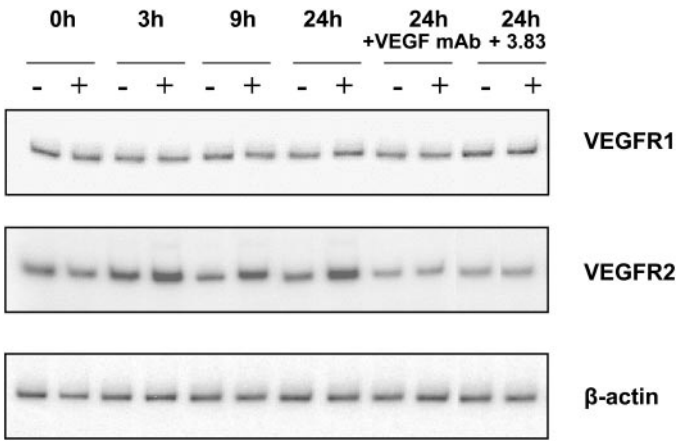

Fig. 5. Absence of effect on VEGFR1 expression and stimulation of VEGFR2 expression by MG63-CM. Expression of VEGFR1 and VEGFR2 mRNA as assessed by RT-PCR is shown. These assays were performed in the presence of MG63-CM (+) or FM (−), in conditions similar to those in Fig. 3. Times indicate length of EC incubation before RNA isolation. Antibodies were used at the same concentrations in Fig. 3. β-Actin was used as internal control. Figure is representative of 3 experiments yielding similar results.

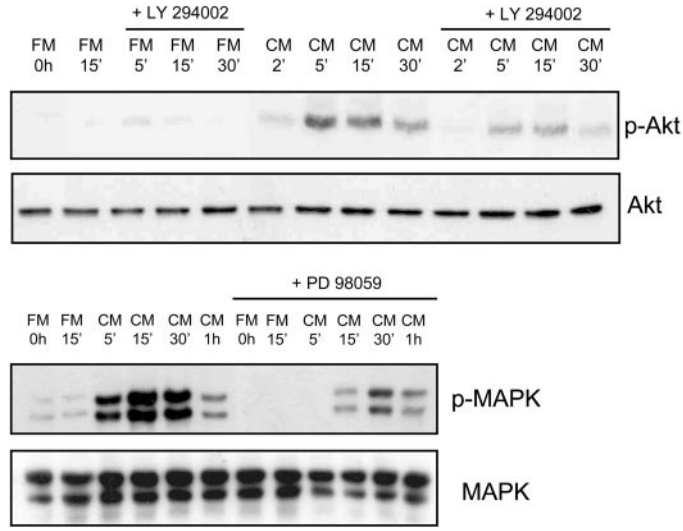

Fig. 6. Effect of MG63-CM on protein kinase B (PKB)/Akt and mitogen-activated protein kinase (MAPK) phosphorylation. Confluent EC were incubated in FM or MG63-CM (CM) for times indicated. Akt and MAPK phosphorylation (p-Akt and p-MAPK respectively), as well as total Akt and MAPK, were determined by Western blotting. A phosphatidylinositol 3-kinase (PI3-kinase) inhibitor (LY-294002; 20 μM) or a MAPK kinase (MEK) inhibitor (PD-98059; 50 μM) was used when appropriate.

The use of anti-VEGF antibodies or drugs as antiangiogenic agents is generally supposed to occur because of the blockade of EC proliferation but not the facilitation of EC damage by tumor-related factors. Therefore, our data extend the scope of putative effects of anti-VEGF therapy in cancer to antiprotective actions on EC. Accordingly, our results add further evidence for the interpretation of the mechanisms of the antiangiogenic effect of VEGF antagonism in tumors and provide a source of explanation for the vessel regression observed on VEGF blockade (2, 3).

The data demonstrating that MG63-CM suppress autologous VEGF gene expression in EC are potentially relevant in terms of interpreting the deleterious effects of VEGF withdrawal or antagonism (2, 3, 11, 16, 25). Tumor-MG63-CM suppresses autologous VEGF, therefore rendering the EC dependent on exogenous, tumor-generated VEGF. Our results agree with findings in human tumors showing the absence of VEGF mRNA in EC from tumors in which VEGF is produced by the neoplastic cells (4, 5, 9, 19). In these, intratumoral hypoxia should have stimulated production of VEGF by the EC within the tumor (25); in fact, the absence of such production suggests that it may actually be suppressed by the VEGF present in the tumor microenvironment. These particular results add new information to previously published work by Wang et al. (24). These authors reported that tumor-CM regulates the expression of VEGF receptors on EC surface, but they did not analyze the fate of autologous VEGF expression by EC (24).

Several of the effects found with the anti-VEGF and anti-VEGFR2 blocking MAbs deserve special comment. First, the

induction of EC growth advantage in reseeding, as well as the induction of Matrigel penetration, indicate that VEGF is a critical factor in triggering properties of the EC that are relevant for the mechanism of metastatic seeding. Second, the different degree of blockade of diverse effects by the anti-VEGF antibody compared with the anti-VEGFR2 antibody suggests either that a more relevant role of VEGFR1 may exist in some of the MG63-CM actions or that other types of VEGF, e.g., VEGF-C or VEGF-D, are involved.

In terms of mechanism, the present results reveal that MG63-CM activates the two main survival pathways, i.e., PI3-kinase/Akt and p44/p42-MAPK. These pathways are involved in the intracellular signaling routes of several growth factors, including VEGF (23). A more precise definition of these routes is beyond the scope of the present study. However, experiments are presently being performed in our laboratory to specifically characterize precise mechanisms involved in the Akt and p44/p42-MAPK activation by MG63-CM.

The findings described herein provide new information for understanding anti-VEGF strategies in the therapy of tumors. The presence of high amounts of exogenous VEGF appears to be critical in allowing the EC to withstand the challenge of deleterious factors produced by the tumor cells. Furthermore, the fact that tumor medium dramatically downregulates autologous VEGF expression in EC may have potential relevance in terms of the response of these EC to forthcoming aggressions. The finding that the interference with VEGF- or VEGFR2-related effects determines a growth disadvantage on reseeding, which interferes with matrix penetration as well, is potentially important for therapeutic approaches against tumor metastatization and local tissue invasion.

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