Modulation of bcl-xL in Tumor Cells Regulates Angiogenesis through CXCL8 Expression

Simona Giorgini,1 Daniela Trisciuglio,1 Chiara Gabellini,1 Marianna Desideri,1 Laura Castellini,1 Cristina Colarossi,2 Uwe Zangemeister-Wittke,3 Gabriella Zupi,1 and Donatella Del Bufalo1

1Experimental Chemotherapy Laboratory, Regina Elena Cancer Institute, and 2Pathology Unit, Sant’ Andrea Hospital, University “La Sapienza,” Rome, Italy; and 3Division of Medical Oncology, University Hospital Zurich, Zurich, Switzerland

Abstract
In this paper, we investigated whether bcl-xL can be involved in the modulation of the angiogenic phenotype of human tumor cells. Using the ADF human glioblastoma and the M14 melanoma lines, and their derivative bcl-xL—overexpressing clones, we showed that the conditioned medium of bcl-xL transfectedants increased in vitro endothelial cell functions, such as proliferation and morphogenesis, and in vivo vessel formation in Matrigel plugs, compared with the conditioned medium of control cells. Moreover, the overexpression of bcl-xL induced an increased expression of the proangiogenic interleukin-8 (CXCL8), both at the protein and mRNA levels, and an enhanced CXCL8 promoter activity. The role of CXCL8 on bcl-xL—induced angiogenesis was validated using CXCL8-neutralizing antibodies, whereas down-regulation of bcl-xL through antisense oligonucleotide or RNA interference strategies confirmed the involvement of bcl-xL on CXCL8 expression. Transient overexpression of bcl-xL led to extend this observation to other tumor cell lines with different origin, such as colon and prostate carcinoma. In conclusion, our results showed that CXCL8 modulation by bcl-xL regulates tumor angiogenesis, and they point to elucidate an additional function of bcl-xL protein. (Mol Cancer Res 2007;5(8):761–71)

Introduction
Angiogenesis is essential for the growth of both primary and metastatic tumors (1), and the understanding of the genetic alterations involved in angiogenic dysregulation could lead to the development of antiangiogenic therapies. Angiogenesis is activated by different factors, including hypoxia, oncogenes, and antioncogenes (2). We have previously shown that bcl-2 overexpression in several human tumor histotypes synergizes with hypoxia to increase angiogenesis through vascular endothelial growth factor (VEGF) expression and hypoxia inducible factor-1 transcriptional activity (3–7). Moreover, treatment of melanoma cells with bcl-2/bcl-xL antisense oligonucleotides reduces angiogenic activity (8). The ability of bcl-2 to induce angiogenesis has also been shown in prostate carcinoma and microvascular endothelial cells (9, 10).

The present study aims to explore the ability of bcl-xL, an antiapoptotic member of the bcl-2 family, to modulate tumor angiogenesis. bcl-xL is a prosurvival multidomain protein that, in addition to promoting cell survival (11), has been implicated in the regulation of cell cycle (12), in the modulation of cell differentiation (13), and in the resolution of inflammation (14). A pivotal role for bcl-xL in breast cancer metastatization (15) and in anaplastic lymphoma kinase—mediated oncogenicity (16) has also been shown. In the present study, we analyzed the functional effect of the modulation of bcl-xL in the angiogenic process of glioblastoma and melanoma.

In this regard, glioblastoma is a particular, suitable model for our study in which vascular changes accompany the advancement of this neoplasia (17), and the expression of bcl-xL is elevated when compared with normal glial or astrocytic cells (18, 19). Recently, the induction of bcl-xL in human glioblastoma cells has been found to confer infiltrative growth in vivo and reduced survival of tumor-bearing mice (20). Moreover, the relevance of bcl-xL in the resistance of glioblastoma to therapy has been widely shown (21, 22).

Furthermore, the role of bcl-xL as an antiapoptotic molecule has been well established also in various melanoma models (23, 24), and the general consensus in the literature is that the bcl-xL protein level seems to increase with melanoma progression (24–26).

In this paper, we show that bcl-xL modulation regulates CXCL8 expression in several tumor cell lines with different histotypes and angiogenesis of melanoma and glioblastoma.

Results
bcl-xL Overexpression in the ADF Glioblastoma and M14 Melanoma Cell Lines Enhances In vivo Angiogenesis

The human ADF glioblastoma and M14 melanoma cell lines were stably transfected with a bcl-xL expression vector or with the vector carrying the neomycin—resistant gene (control clone). About 5-fold increase of bcl-xL protein expression was
observed in bcl-xL–overexpressing clones (AXL42, AXL74) when compared with the parental glioblastoma cells (ADF) or the control clone (AN8; Fig. 1A). As reported in Fig. 1A, an undetectable level of bcl-xL protein was observed in M14 parental and control (Mneo) cells under our experimental condition, whereas both bcl-xL transfectants (MXL12, MXL90) expressed a consistent level of bcl-xL protein.

To examine whether bcl-xL plays a role in the angiogenesis of glioblastoma and melanoma, in vivo vessel formation in Matrigel was examined (Fig. 1B–E). Matrigel plugs containing the conditioned medium (CM) of control or bcl-xL–overexpressing clones were injected in mice, and the degree of vascularization into Matrigel plugs was evaluated. As evidenced by macroscopic analysis, CM of the glioblastoma (AN8) and melanoma (Mneo, M14) control cells induced only a slight angiogenic response compared with the negative control (NEG), the CM is replaced with SFM. C and E. Measurement of hemoglobin content in each Matrigel plug expressed as absorbance (OD) per gram of Matrigel plug. P values are reported in the text. Western blot analysis of bcl-xL protein in the ADF glioblastoma line and its derivative control (AN8) and bcl-xL–overexpressing (AXL42, AXL74) clones and in the M14 melanoma line and its derivative control (Mneo) and bcl-xL–overexpressing clones (MXL12, MXL90). Western blot analysis representative of two independent experiments with similar results are shown.
synthesis of endogenous mediators of angiogenesis, we evaluated the expression of several angiogenic factors through a human angiogenesis protein array (Fig. 3). A schematic representation of the proangiogenic factors that can be detected by the use of the array was reported in Fig. 3C. An increased secretion of CXCL8 was observed in both glioblastoma (about 1.5-fold induction; Fig. 3A) and melanoma (from 1.5- to 3-fold induction; Fig. 3B) lines after bcl-xL overexpression. To confirm the results obtained with the protein array, the level of CXCL8 protein in the CM of bcl-xL transfectants and control cells was measured (Figs. 4 and 5). A significant increase in the amounts of secreted CXCL8 was observed when bcl-xL–overexpressing glioblastoma cells were compared with control cells: about 3-fold increase in CXCL8 protein secretion was evident after both 24 h (AXL42, $P = 0.031$; AXL74, $P = 0.029$) and 48 h (AXL42 and AXL74, $P = 0.009$) of cell culture (Fig. 4A). Figure 4B shows Northern blot analysis of CXCL8 mRNA expression in control (AN8) cells and AXL42 bcl-xL transfectant: the AN8 control cells expressed a slightly detectable level of CXCL8 transcript, whereas CXCL8 mRNA was clearly evident in the bcl-xL transfectant exposed to the same experimental condition. The effect of bcl-xL on the expression of other chemokines and angiogenic factors that are involved in the growth of glioblastoma was also evaluated (Fig. 4C). The expression of transforming growth factor-$\beta_1$ (TGF-$\beta_1$) and VEGF proteins that were not detectable either in parental and bcl-xL–overexpressing clones using the protein array was evaluated by ELISA assay. As reported in Fig. 4C, no difference in the levels of both proteins was observed when comparing CM from bcl-xL–overexpressing cells to control clones. We also analyzed by ELISA the secretion of interleukin-1$\beta$ (IL-1$\beta$) protein, an angiogenic factor, and TGF-β2, a molecule recently shown to be regulated by bcl-xL overexpression in glioma cells (20). The antibodies for these two proteins were not included in the protein membrane. As reported in Fig. 4C, TGF-β2 protein was found to be equally secreted in parental and bcl-xL–overexpressing cells, whereas IL-1$\beta$ was not detectable both in control and bcl-xL transfectants at the experimental condition used. Because from the protein array bcl-xL seems to modulate tissue inhibitor of metalloproteinase 1 (TIMP1) and TIMP2 in glioblastoma cells, we also tested the expression of these two proteins by Western blot analysis: no differences in TIMP1 and TIMP2 expression were observed between parental and bcl-xL–overexpressing cells (data not shown).

Regarding the effect of bcl-xL overexpression on the expression of angiogenic factors by melanoma cells, whereas no significant differences in VEGF secretion (Fig. 5C) or expression (Fig. 5D) were observed between control cells and bcl-xL–overexpressing clones, a significant difference in CXCL8 secretion was evidenced by ELISA in time course experiments (Fig. 5A). This difference was already evident 12 h after cell culture: CXCL8 was detected at a low level in parental cells (100 ± 5.3 pg/10$^6$ cells), whereas about

**FIGURE 2.** bcl-xL overexpression in ADF glioblastoma and M14 melanoma cells increases in vivo vessel formation in a Matrigel assay. Representative pictures of histologic analysis in Matrigel plug sections. Matrigel plugs containing CM from bcl-xL–overexpressing cells (AXL42 and AXL74 glioblastoma; MXL12 and MXL90 melanoma) reveal a marked capillary vessel proliferation, as compared with Matrigel plugs containing CM from control cells (AN8 glioblastoma and Mneo melanoma). In the negative and positive controls, the CM is replaced with SFM or angiogenic factors (8), respectively (Masson’s trichrome staining; original magnification, 10×).
1,000 ± 47.7 pg/10^6 cells of CXCL8 were secreted by bcl-xL–overexpressing cells (MXL12, \( P = 0.041 \); MXL90, \( P = 0.036 \)). This difference was maintained until 72 h of cell culture (MXL12, \( P = 0.035 \); MXL90, \( P = 0.029 \)). Western blot analysis of CM confirmed the higher CXCL8 protein secretion in bcl-xL transfectants compared with the control cells (Fig. 5B). As reported for glioblastoma, no modulation of TGF-\( \beta \) was observed after forced expression of bcl-xL in melanoma cells (Fig. 5C), whereas TGF-\( \beta \)-1 and IL-1\( \alpha \) were not detectable both in control and bcl-xL transfectants.

**bcl-xL Overexpression Increases In vitro and In vivo Angiogenesis through CXCL8**

Several evidences indicate that CXCL8 is an angiogenic factor (27, 28). Thus, we analyzed whether the increase in CXCL8 secretion induced by bcl-xL was responsible for the enhancement of angiogenesis we previously observed in bcl-xL–overexpressing cells (Fig. 1). In particular, to validate the role of CXCL8 on bcl-xL–induced angiogenesis, we evaluated in vitro endothelial cell functions, such as proliferation and morphogenesis, in the presence of CM from different glioblastoma lines containing CXCL8-neutralizing antibodies or not. Figure 6A shows the proliferation of human umbilical vascular endothelial cells (HUVEC) in the presence of CM obtained after 48 h of cell culture. The CM from the bcl-xL–overexpressing clones induced about a 2-fold increase of HUVEC proliferation (AXL42 and AXL74, \( P = 0.001 \)) when compared with CM from the AN8 control clone. Addition of CXCL8-neutralizing antibodies to the CM from bcl-xL transfectants reduced HUVEC proliferation by about 40% (AXL42, \( P = 0.005 \); AXL74, \( P = 0.001 \)) to the level observed with CM from control cells. VEGF-neutralizing antibodies did not induce a significant inhibition of cell proliferation.

Next, we examined HUVEC capillary sprouting in the presence of CM from the different glioblastoma lines. As reported in Fig. 6B, HUVEC seeded on Matrigel in the presence of CM from bcl-xL transfectants developed capillary-like sprouts throughout the Matrigel surface; on the contrary, endothelial cells exposed to CM from the control cells showed only a reduced spread. In particular, 3 ± 2 sprouts per field were counted for HUVEC exposed to CM from the AN8 clone, whereas 13 ± 5 and 15 ± 3 sprouts per field were counted for HUVEC exposed to CM from AXL42 (\( P = 0.007 \)) and AXL74 (\( P = 0.005 \)), respectively. Moreover, whereas VEGF-neutralizing antibodies added to CM from bcl-xL transfectants did not induce a significant decrease of HUVEC morphogenesis, addition of CXCL8-neutralizing antibodies reduced the number of sprouts in HUVEC exposed to CM from bcl-xL transfectants by about 80% (AXL42 and AXL74, \( P = 0.001 \); Fig. 6B).
In addition, the angiogenic response in Matrigel plugs in the presence of CM from both melanoma and glioblastoma cells overexpressing bcl-xL was markedly reduced by adding specific neutralizing antibodies directed to CXCL8. The hemoglobin content in the Matrigel plugs containing CM from the bcl-xL transfectant was reduced by 70% (AXL42, \( P = 0.009 \); AXL74, \( P = 0.008 \)) and 55% \( (P = 0.038 \) when using CXCL8-neutralizing antibodies in glioblastoma (Fig. 6C) and melanoma (Fig. 6D), respectively.

**bcl-xL Overexpression Induces CXCL8 Secretion in Several Glioblastoma Cell Lines**

We next evaluated the effect of bcl-xL overexpression on several human glioblastoma cell lines. Figure 7A shows Western blot analysis of bcl-xL protein expression in LI, T98G, U373MG, CRS-A2, and U138 glioblastoma lines: bcl-xL was expressed in all the lines analyzed. Moreover, in those lines expressing high levels of bcl-xL, such as LI, U373MG, and T98G, CXCL8 secretion was found to be higher (respectively, \( 3.348 \pm 225, 2.005 \pm 1 \), and \( 1.763 \pm 104 \) pg of CXCL8) than CRS-A2 and U138, expressing undetectable levels of CXCL8 and low levels of bcl-xL. Then, we evaluated whether transient overexpression of bcl-xL was able to increase CXCL8 expression in the ADF and in other glioblastoma lines (U138, CRS-A2, U373MG, and T98G). As reported in Fig. 7B, an increase in CXCL8 expression of about 1.7-fold (U373MG, \( P = 0.042 \); T98G, \( P = 0.036 \)) and 2.7-fold (ADF, \( P = 0.029 \); CRS-A2, \( P = 0.026 \)) and 3.9-fold (U138, \( P = 0.008 \) was observed after bcl-xL overexpression, thus confirming the effect of bcl-xL on CXCL8 secretion in ADF and extending this result to four other glioblastoma lines. Because gene transcription is one major point of control at which expression of CXCL8 is regulated (29), we next determined whether the increase in CXCL8 secretion induced by bcl-xL overexpression was due to a transcriptional up-regulation of the corresponding gene (Fig. 5). To this end, luciferase reporter construct containing wild-type CXCL8 proximal promoter was cotransfected with the control vector PEQ-176 into control cells (AN8 and Mneo) and bcl-xL transfectants from glioblastoma (AXL42 and AXL74, Fig. 7C). As shown in Fig. 7C, basal activation of CXCL8 promoter region was up-regulated of about 2-fold in the glioblastoma (AXL42, \( P = 0.025 \); AXL74, \( P = 0.031 \)) bcl-xL-overexpressing clones when compared with control clones. U138 glioblastoma line was also used to confirm the ability of bcl-xL to regulate CXCL8 promoter activity. As shown in Fig. 7D, the transient expression of increasing amounts of bcl-xL expression vector (from 0.25 to 0.75 \( \mu \)g) in this line induced a significant increase of CXCL8 promoter activity (from about 1.7-fold to 4.5-fold, depending on the amount of bcl-xL vector used; 0.25 \( \mu \)g, \( P = 0.005 \); 0.5 and 0.75 \( \mu \)g, \( P = 0.001 \)) as compared with the bcl-xL basal promoter activity.

**bcl-xL Overexpression Induces CXCL8 Secretion in Some Tumor Cell Lines of Different Origin**

CXCL8 promoter activity after bcl-xL overexpression was also investigated in melanoma cells. As shown in Fig. 8A, basal activation of CXCL8 promoter region was up-regulated of about 1.5-fold in melanoma (MXL90, \( P = 0.037 \); MXL12, \( P = 0.038 \)) bcl-xL–overexpressing clones when compared with control clones. bcl-xL protein was also found to be expressed in several human melanoma lines, although different expression levels were observed (Fig. 8B). Moreover, transient overexpression of bcl-xL was also able to increase CXCL8 expression (from 1.4- to 1.7-fold) in the M14 (\( P = 0.042 \)), LP (\( P = 0.041 \)), SbCl1 (\( P = 0.038 \)), and PLF2 (\( P = 0.033 \)) melanoma lines (Fig. 8C). An increase in CXCL8 secretion after transient overexpression of bcl-xL was also shown in human colon (LoVo, 4.2-fold induction, \( P = 0.007 \)) and prostate (PC3, 2.0-fold induction, \( P = 0.036 \)) carcinoma cells (Fig. 8D), two tumor histotypes with a high expression level of bcl-xL protein (30, 31), thus confirming the capability of bcl-xL to modulate CXCL8 expression to tumor cells with different origins. Despite a variability in bcl-xL protein expression from line to line, Western blot analysis of bcl-xL protein expression shows high levels of bcl-xL protein in two colon and two prostate carcinoma lines (Fig. 8E).

**Down-Regulation of bcl-xL Expression Reduces CXCL8 Expression**

To confirm the role of bcl-xL in the regulation of CXCL8 expression, bcl-xL protein expression was down-regulated through antisense oligonucleotide (AS) or RNA interference
strategies. Western blot analyses (Fig. 9A and C) showed that treatment with AS specific for \textit{bcl-xL} decreased \textit{bcl-xL} expression both in the AXL74 \textit{bcl-xL} transfectant from glioblastoma (about 60% inhibition, Fig. 9A) and MXL12 \textit{bcl-xL} transfectant from melanoma (about 40% inhibition, Fig. 9C). Down-regulation of CXCL8 protein expression was also induced by \textit{bcl-xL} AS treatment: ELISA assays evidenced the decrease of CXCL8 protein secretion after AS treatment both in AXL74 (about 70% inhibition, \(P = 0.009\), Fig. 9B) and MXL12 (about 40% inhibition, \(P = 0.007\), Fig. 9D) \textit{bcl-xL}–overexpressing clones. The control sequence (SC) did not affect \textit{bcl-xL} or CXCL8 expression (Fig. 9A–D). Similar results were obtained using AXL42 glioblastoma and MXL90 melanoma transfectants (data not shown). On the contrary, the decrease of \textit{bcl-xL} expression by AS exposure did not significantly modify the level of secreted VEGF in both histotypes (data not shown).

We also evaluated whether \textit{bcl-xL} down-regulation by \textit{bcl-xL} small interfering RNA (siRNA) in U373MG glioblastoma cells (Fig. 9E) and MXL12 melanoma \textit{bcl-xL} transfectant (Fig. 9G) was able to affect CXCL8 expression: a reduction of \textit{bcl-xL} expression by \textit{bcl-xL} siRNA in both histotypes (Fig. 9E and G) was paralleled by a decrease of CXCL8 protein secretion of about 50% in glioblastoma (\(P = 0.008\), Fig. 9F) and of about 40% in melanoma (\(P = 0.016\), Fig. 9H). The control siRNA did not modify CXCL8 secretion (Fig. 9F and H). These results confirm a relationship between \textit{bcl-xL} and CXCL8 expression.

Discussion

The major finding of our study is that \textit{bcl-xL} is able to modulate CXCL8 expression in some human cancer cell lines. In addition, \textit{bcl-xL}–mediated-CXCL8 induction increases angiogenesis in glioblastoma and melanoma cells. In particular, stable \textit{bcl-xL} overexpression in both tumor histotypes induced CXCL8 protein secretion, mRNA expression, and promoter activity. Moreover, CM from \textit{bcl-xL} transfectants enhanced \textit{in vitro} proliferation and morphogenesis of endothelial cells and \textit{in vivo} vessel formation in a Matrigel assay. Enhancement of CXCL8 expression induced by \textit{bcl-xL} was dependent on gene transcription: transient transfection experiments with wild-type CXCL8 promoter reporter construct showed that \textit{bcl-xL} overexpression increased CXCL8 promoter activity in glioblastoma and melanoma lines. A decreased secretion of CXCL8 protein was observed after the down-regulation of \textit{bcl-xL} protein expression using \textit{bcl-xL}–specific AS or siRNA, and the use of CXCL8–specific neutralizing antibodies validated the role of CXCL8 on \textit{bcl-xL}–induced angiogenesis. Transient transfection experiments confirmed the ability of \textit{bcl-xL} to increase CXCL8 protein expression not only in the glioblastoma and melanoma lines, but also in tumor cell lines of different origin such as prostate and colon carcinoma, thus indicating that the CXCL8 induction by \textit{bcl-xL} is not a phenomenon restricted to glioblastoma and melanoma, but it could be extended to other kinds of tumors.

We excluded the possibility that \textit{bcl-xL} could increase angiogenesis in our models through the enhancement of other important angiogenic factors or chemokines involved in the angiogenic phenotype, such as VEGF, TGF-\(\beta\), IL-1\(\beta\), TIMP1, and TIMP2, because no modulation of these proteins was observed after \textit{bcl-xL} overexpression.

There are several novel aspects in our study. It is the first to show that \textit{bcl-xL} overexpression in several tumor histotypes...
enhances CXCL8 expression and, in glioblastoma and melanoma, determines an increase of CXCL8-induced angiogenesis. These data suggest that, in addition to its antiapoptotic function, bcl-xL may also play a role in angiogenesis by inducing CXCL8 secretion. Because bcl-2 overexpression in experimental tumor models increases angiogenesis through the enhancement of VEGF expression (4, 5, 9) and forced expression of bcl-2 in microvascular endothelial cells increases intratumoral angiogenesis and accelerates tumor growth through CXCL8 (10), beyond their ability to block apoptotic signals, bcl-2 and bcl-xL share other important functions, such as modulation of the angiogenic phenotype.

The relevance of CXCL8 in glioblastoma has been reported in several studies: CXCL8 is expressed and secreted at high levels both in vitro and in vivo, and it is critical for glial tumor neovascularity and progression (33). Moreover, the levels of CXCL8 correlate with histologic grade in glial neoplasms. Increased CXCL8 in glioblastoma occurs in response to various stimuli, including oncogene activation (34, 35). A time-dependent dissociation between the modulation of apoptosis and the invasiveness of cells has been also reported after bcl-xL overexpression in human malignant glioma cells (22).

CXCL8 has also been proposed to play a key role in melanoma growth and metastasis: increased serum concentration of CXCL8 in malignant melanoma patients correlates with tumor progression and survival (36), and expression of CXCL8 correlates with aggressiveness (37) and worse prognosis in primary cutaneous melanoma (38).

Numerous studies have suggested that the dysregulation of the apoptotic program could favor not only tumor resistance to treatment but also tumor progression or initiation of oncogenesis (39, 40). Very recently, it has been found that bcl-xL is required for the tumorigenic conversion of immortalized primary human cells (38). Moreover, a correlation between bcl-xL and tumor progression has been described in different tumor histotypes. In particular, it has been shown that bcl-xL expression is able to modulate the tumorigenic potential and promote metastatization in lymphoma (26) and in breast carcinoma (25). The results reported in our study sustain the growing body of evidence indicating a positive role of bcl-xL in tumor progression (25, 26) and suggesting bcl-xL as a potential target for glioblastoma and melanoma treatment.

In conclusion, the up-regulation of bcl-xL has several major consequences for the growth of glioblastoma and melanoma.
As shown by several papers, it inhibits apoptosis induced by several stimuli, improving the ability of tumor cells to escape from therapy (12, 13). Moreover, as reported in this study, bcl-xL overexpression increases the expression of CXCL8, an endogenous inducer of in vitro and in vivo angiogenesis (28). These functions have a direct and significant influence on the glioblastoma and melanoma progression and angiogenesis. Thus, the blockade of bcl-xL protein expression by AS treatment or chemical selective inhibitors could be used to inhibit the angiogenic potential and improve the treatment of these neoplasia.

Materials and Methods

Cell Lines, Transfections, Oligonucleotide, and siRNA Treatment

Human glioblastoma (ADF, CRS-A2, LI, T98G, U373MG, and U138), melanoma (M14, PLF2, SbCl1, LP, M20, ME1007, MEL120, andJR1), prostate carcinoma (PC3 and DU145), colon carcinoma (LoVo and HT29), umbilical vein endothelial (HUVEC) cell lines, two bcl-xL–overexpressing clones (AXL42 and AXL74), and a control clone (AN8) derived from the ADF line after stable transfection, two bcl-xL–overexpressing clones (MXL90 and MXL12) and a control clone (Mneo) derived from the M14 line after stable transfection were used.

Stable and transient transfections with bcl-xL expression vector (pcDNA3-bcl-xL), bcl-xL antisense oligonucleotides (AS, 750 nmol/L, ref. 41) or bcl-xL siRNA (100 nmol/L, 5’-GGAGAUGGCAGGUUAUUGUGUU-3’, Dharmacon Research) were done using LipofectAMINE or lipofectin (Invitrogen). After transient transfections, cells were exposed to serum-free medium (SFM) for 24 h, and then adherent cells and CM were differentially processed according to the analyses to be done. Control experiments were done using scrambled oligonucleotides (SC, 100 nmol/L, 5’-CACGTCACGCGCGCACTATT-3’, Dharmacon Research; ref. 41), siRNA against unrelated mRNA, or empty expression vectors.

Western Blot Analysis

Proteins from cellular lysates or CM were fractionated by SDS-PAGE, transferred to a nitrocellulose filter, and subjected to immunoblot assays. Antibodies against bcl-xL and TIMP2 (Santa Cruz Biotechnology), VEGF and HSP 72/73, TIMP1 (Chemicon International), and β-actin (Sigma) were used. Red Ponceau (Sigma) staining of filter and β-actin or HSP 72/73 protein amount were used to check equal loading and transfer of proteins.

ELISA and Human Angiogenesis Antibody Array

ELISA assay (R&D Systems) was used to evaluate the level of secreted CXCL8, VEGF, IL-1β, TGF-β1, and TGF-β2 in CM. The sensitivity of the assay was 31.2 pg/mL for CXCL8 and TGF-β1, 15.6, 7.0, and 3.9 pg/mL for VEGF, TGF-β2,
and IL-1β, respectively. About 5 × 10^5 cells were seeded in 100-mm plates and, 24 h later, exposed to SFM for 12, 24, 48, and 72 h. Proteins were normalized to the number of adherent cells counted at the time of collection.

The Human Angiogenesis Antibody Array I (RayBiotech. Inc.) was used according to the manufacturer’s protocol to evaluate the secretion of 20 angiogenic factors into the CM of the different lines. A schematic representation of the proangiogenic factors that may be detected by the use of the array has been reported in Fig. 3C. Membranes spotted in duplicate with antibodies against angiogenic factors were blocked with blocking buffer and then were incubated overnight with CM. Next, membranes were washed with wash buffer, incubated with biotin-conjugated antibodies against proangiogenic factors, washed with wash buffer, and incubated with horseradish peroxidase–conjugated streptavidin. The signals on the membranes were detected by chemiluminescence. Membranes, blocking and wash buffer, and antibodies against proangiogenic factors were all provided with the kit. The intensity of protein signal (two spots for each protein) was compared with the relative positive signals by densitometric analysis.

**Northern Blot Analysis**

Total RNA was prepared, and Northern blot analysis was done as previously described (4) using a 298-bp fragment specific for human CXCL8 mRNA.

**Promoter Activity**

To study CXCL8 promoter activity, 0.8 to 1 × 10^5 cells per well (24-well plate) were seeded in triplicate and, 24 h later, were transfected using LipofectAMINE with an internal control PEQ-176 plasmid (0.1 µg) and the luciferase reporter vector pUHC13-3 (0.4 µg) carrying wild-type CXCL8 promoter (nucleotides 1348–1527 of the CXCL8 gene, ref. 42). To study the effect of transient bcl-xL overexpression on wild-type CXCL8 promoter activity, different amounts of bcl-xL expression vector (from 0.25 to 0.75 µg) were transfected in quadruplicate with the reporter vectors described above.

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**FIGURE 8.** bcl-xL overexpression in several tumor cell lines induces CXCL8 secretion and promoter activity. **A.** CXCL8 promoter activity in the melanoma parental (M14), control (Mneo), and bcl-xL–overexpressing (M XL90 and M XL12) cells. **B.** Western blot analysis of bcl-xL expression in several human melanoma cell lines. **C.** ELISA assay of CXCL8 secretion in CM after transient transfection of melanoma cell lines with control (filled columns) or bcl-xL expression (open columns) vectors. **D.** ELISA assay of CXCL8 secretion in CM after transient transfection with control (filled columns) or bcl-xL expression (open columns) vectors of LoVo colon and PC3 prostate carcinoma cell lines. **E.** Western blot analysis of bcl-xL expression in colon (LoVo and HT29) and prostate (DU145 and PC3) carcinoma cell lines. **A, C, and D.** Columns, mean of three independent experiments done in triplicate; bars, SD. Fold induction of CXCL8 protein (C and D) or promoter activity (A) relative to the basal one (filled columns). P values are reported in the text. *, P < 0.05; **, P < 0.01. **B** and **E.** Western blot analysis representative of two independent experiments with similar results are shown.

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The luciferase activity of each sample was normalized to β-galactosidase activity to calculate relative luciferase activity.

Densitometric Analyses

Developed films were acquired using GS-700 Imaging Densitometer (Bio-Rad) and processed with Corel Photo Paint 7.0 to adjust image brightness and contrast. Densitometric evaluation was done using Molecular Analyst Software (Bio-Rad) and normalized with relative controls depending on the analysis done.

In vitro Proliferation and Morphogenesis of HUVEC

Exponentially growing HUVEC were seeded for proliferation (7 x 10^3 cells per 96-well microtiter plate) or morphogenesis (1.5 x 10^4 cells per 96-well microtiter plate containing polymerized Matrigel) and incubated with CM obtained from control cells or from bcl-xL transfectants, alone or in the presence of specific human CXCL8- or VEGF-neutralizing antibodies (anti-CXCL8 and anti-VEGF, 0.2 µg/mL, R&D Systems) or normal mouse immunoglobulin G (Pierce Biotechnology, Inc.) as control. Cells incubated in the presence of rhCXCL8 (10 ng/mL, R&D Systems) were used as positive control. Cell proliferation was evaluated after 48 h by a colorimetric assay as previously described (5) and reported as absorbance. In morphogenesis experiments, the number of sprouts in six random microscopic fields was counted in triplicate wells at 10 x original magnification, using the inverted phase contrast microscope Leitz Fluovert (Wetzlar).

In vivo Matrigel Assay

In vivo Matrigel assay and quantification of the hemoglobin content in the Matrigel plugs were done as previously reported (4) using cell suspensions (5 x 10^6 viable cells) or CM (10 x concentrated) in the absence or presence of CXCL8-neutralizing antibodies (5 µg/mL). Groups of eight mice were used for each experimental point. All procedures involving animals and their care were conducted in conformity with the institutional guidelines, which are in compliance with national and international laws (4). For histologic analysis, Matrigel plugs were fixed with 10% formalin solution and paraffin-embedded. Sections 5 µm thick were deparaffinized and stained with Masson’s trichrome according to the manufacturer’s protocol.
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(Boihtica Spa). In the negative and positive controls, the CM was replaced with SFM or angiogenic factors, respectively.

Statistical Analysis

Results were expressed as the mean ± SD from n determinations. The significance of differences between the means was determined with Student’s two-tailed t test, done with SPSS software. Values of P < 0.05 were accepted as statistically significant.

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