Unexpected properties of endostatin-producing mouse *BCR-ABL*-transformed cells

MONIKA KRMENČÍKOVÁ[1], LIBOR STANĚK[1,2], MARTINA PETRÁČKOVÁ[1], PAVEL DUNDR[2] and VLADIMÍR VONKA[1]

[1]Department of Experimental Virology, Institute of Hematology and Blood Transfusion, U Nemocnice 1, 128 20 Prague 2; [2]Institute of Pathology, General Faculty Hospital, Studnickova 2, 128 00 Prague, Czech Republic

Received July 27, 2011; Accepted September 2, 2011

DOI: 10.3892/ijo.2011.1213

Abstract. We investigated whether a genetic modification of *BCR-ABL*-transformed mouse cells that resulted in endostatin (ES) production altered their oncogenic potential. Mouse B210 cells, which express $p210^{bcr-abl}$ fusion protein and induce leukemia-like disease and extremely rarely solid tumors after intravenous (i.v.) administration, were used. The cells were transfected with a plasmid carrying genes for mouse ES and resistance to blasticidine. Transduced cells were isolated in media supplemented with blasticidine. Production of ES was determined by Western blotting. For further tests, two clones were selected, and their pathogenicity after i.v. inoculation was tested. Compared with the parental B210 cells, the capability of both gene-modified cell clones to induce lethal leukemia was reduced. However, mice that did not succumb to leukemia subsequently developed solid tumors. They were composed of poorly differentiated cells with irregular nuclei and roughly granular chromatin and were well vascularized. FISH revealed the presence of the *BCR-ABL* fusion gene both in tumors and spleens. Immunohistological investigation of the tumors demonstrated the production of ES *in vivo* and the cell lines derived from the tumors produced detectable amounts of ES, this demonstrating that the formation of solid tumors was not associated with the loss or silencing of the ES gene.

Introduction

Tumor growth and the formation of metastases depend on the development of a new network of vasculature that supplies the tumors with nutrients and oxygen and removes metabolic waste. There is evidence that angiogenesis is needed not only for the growth of solid tumors but also for the development of hematological malignancies such as chronic myeloid leukemia (1). Angiogenesis, a principal determinant of tumor growth (2) is a very complex process and an ever increasing number of substances with either pro- or anti-angiogenic activity are being recognized. It is believed that the abundant neoplastic vasculature results from the shift of balance to a more pro-angiogenic state, which is different from the classical one both in its structure and behavior. The use of anti-angiogenic substances seems promising in the therapy of cancer (3,4). Among the endogenously formed substances recognized as suppressors of angiogenesis, attention has mostly concentrated on endostatin (ES), a proteolytic 20-kDa fragment of collagen XVIII which has already been used in clinical trials (5). Although it is known that ES inhibits endothelial cell proliferation and migration, promotes apoptosis and induces growth arrest, the precise molecular mechanism of its action has not been clarified as yet. Because of the broad range of molecular targets for ES, it is believed that numerous signalling pathways are involved in mediating its activities (6). Its ability to reduce experimental tumor growth and metastazing has repeatedly been demonstrated (7-9). In the previous experiments of ours we showed that gene modification of two human papillomavirus type 16 (HPV16)-transformed mouse cell lines that resulted in ES production strongly reduced the oncogenicity and metastizing ability of one but not of the other cell line tested and that the loss of oncogenicity was associated with suppression of interleukin 1α (IL1α) production (10). The aim of our present study was to examine the effects of the same genetic modification on the behavior of BCR-ABL-transformed mouse B210 cells. These studies are a part of our efforts to develop experimental vaccines against *BCR-ABL*-transformed cells and to investigate certain mechanisms which might be useful in strengthening the efficacy of such preparations (11).

Correspondence to: Professor Vladimír Vonka, Department of Experimental Virology, Institute of Hematology and Blood Transfusion, U Nemocnice 1, 128 20 Prague 2, Czech Republic
E-mail: vonka@uhkt.cz

Key words: BCR-ABL, endostatin, solid hematological tumors

Materials and methods

Cell lines and media. Mouse (Balb/c) B210 cells transformed by the *BCR-ABL* fusion gene, were the same as in previous experiments (12-14). These cells had been derived from the Ba/F3 cells of an early B lineage by a retroviral vector carrying the human *BCR-ABL* fusion gene (15). They produce the $p210^{bcr-abl}$ protein and have down-regulated expression of MHC class I molecules. After intravenous (i.v.) administration they induce leukemia-like disease in syngeneic animals and very rarely intraabdominal solid tumors (in <1% of inoculated animals) (Sobotkova and Lučansky, unpublished data). B210 cells and their sublines (see

below) were cultivated in RPMI-1640 medium (Sigma-Aldrich Corp., St. Louis, MO) supplemented with 10% heat-inactivated FCS (PAA Laboratories, Linz, Austria), 2 mmol/l glutamine and antibiotics. In some experiments another *BCR-ABL*-transformed mouse cell line, designated 12B1 (16), was used as a control. These cells, kindly provided by E. Katsanis (University of Arizona) are also of an early B lineage, produce the $p210^{bcr\text{-}abl}$ fusion protein in amounts comparable to B210 cells, but differ from them in a number of properties (14,17) (Krmenčíková *et al*, unpublished data). 12B1 cells were cultivated in RPMI-1640 medium supplemented with 10% heat-inactivated FCS and enriched with 1 mmol/l sodium pyruvate and 50 μmol/l 2-mercaptoethanol. Transfected B210 cells were cultivated in medium supplemented with 15 μg/ml blasticidine (InvivoGen, San Diego, CA). Human umbilical-vein endothelial cells (HUVEC, Cascade Biologics, UK) were incubated in Medium 200 supplemented with LSGS Kit (Cascade Biologics, UK). TE-2 cells, an ES-producing cell line derived from HPV16-transformed TC-1 cells (10), used as a positive control in some tests, were cultivated in D-MEM medium (PAA Laboratories, Linz, Austria) supplemented with 10% FCS and antibiotics. All cell lines were cultivated at 37˚C in a 5% CO_2 atmosphere.

Plasmids and transfection experiments. Plasmid pBLAST49-mEndo (InvivoGen), carrying the genes for mouse ES and for blasticidine resistance was used. It was propagated in *E. coli* strain SURE: e14 (McrA) in TB medium containing 0.1 mg/ml blasticidine (Fast Media Blas Liquid, Cayla-InvivoGen) and was purified using the Qiagen Plasmid Maxi Kit (Qiagen, Germany). Counts of $5x10^6$ B210 cells were resuspended in 500 μl of electroporation buffer (10% RPMI-1640 medium without FCS) and put into an electroporation cuvette (Bio-Rad, Hercules, CA). After adding 15 μg of plasmid DNA, the cells were kept for 10 min at room temperature. They were then shocked once at 280 V, capacitance 1050 μF, at room temperature. The Gene Pulser® Electroporation system (Bio-Rad) was used. The cells were then kept for 10 min at room temperature and transferred into tissue culture dishes each containing 4 ml of complete RPMI medium. After 48 h the transfected cells were transferred into the selection medium supplemented with blasticidine (15 μg/ml). Production of ES was determined by Western blotting (WB) (see below).

Cell cloning. Transduced cells were diluted to a final concentration of 0.3 cell/0.2 ml in a mixture of complete RPMI medium and filtered conditioned medium (2:1). The cell suspension was pipetted into a 96-well plate, 0.2 ml per well (TPP, Trasadingen, Switzerland). Four hours later we marked the wells containing one cell. After 14-day incubation the cell suspensions were transferred into dishes. Large stocks of the individual cloned cell lines were prepared and kept frozen in liquid nitrogen until investigation. In all experiments, the third passage of re-thawed cells was used.

Detection of ES and IL1α by WB. The presence of ES was monitored either in cell lysates or in concentrated tissue culture media. When cell lysates were tested, counts of $12x10^6$ cells were harvested and lysed in 600 μl of lysis buffer containing 4% SDS, 20% glycerol, 10% mercaptoethanol, 2 mM EDTA, 100 mM Tris-Cl, pH 8.0. In the other case, counts of $1x10^6$ cells were seeded in tissue culture dishes in 5 ml RPMI and cells were incubated for 48 h. The media were harvested and concentrated (20x) by using Amicon® Ultra centrifugal filters (Milipore, USA); buffer (1:5) containing 4% SDS, 20% glycerol, 10% mercaptoethanol, 2 mM EDTA, 100 mM Tris-Cl, pH 8.0 was then added. Polyclonal goat anti-mouse ES antibody (Sigma-Aldrich) and horseradish peroxidase-linked rat anti-goat IgG (Amersham Biosciences, UK) were used. Anti-c-Abl (Ab-3) mouse monoclonal antibody (Merck KGaA, Darmstadt, Germany) and horseradish-linked peroxidase anti-mouse IgG antibody (GE Healthcare Biosciences, PA, USA) were used for the detection of bcr-abl fusion protein. IL-1α was detected by means of polyclonal rabbit anti-mouse IL-1α antibody (Abcam, Cambridge, UK) and horseradish peroxidase-linked donkey anti-rabbit IgG antibody (Amersham). The reactions were visualized with the ECL Plus Western Blotting Detection System (GE Heatlth Biosciences, PA, USA).

Flow cytometry for MHC Class I detection. Counts of $5x10^5$ cells were washed twice with washing buffer (0.09% NaN_3, 2% FCS in PBS). Cells were treated with FITC-conjugated anti-mouse $H\text{-}2K^dD^d$ monoclonal antibody (Cedarlane, Hornby, Ontario, Canada) or isotype control antibody (Sigma, St. Louis, MO) for 30 min at 4˚C. Cells were washed and measured on a Coulter Epics XL flow cytometer (Beckman Coulter, Fullerton, CA, USA) and analysed by WinMDI 2.8 software. MHC class I-positive 12B1 cells served as a control.

HUVEC proliferation assay. The procedure used was the same as described previously (10). In brief, 20x concentrated media from the cultures of the various cell lines were used. Counts of $1x10^6$ parental B210 cells or endostatin-producing B210 cells were incubated in 5-ml RPMI medium in 6-cm dishes and cultivated for 48 h. Their media were then withdrawn and concentrated. HUVEC cells were pipetted into 96-well plates; 100 μl of media contained $2x10^3$ cells per well. The next day the medium from HUVEC cultures was replaced with a mixture of fresh regular HUVEC medium (90 μl) and concentrated media (10 μl) from the cultures tested. Every 24 h the media were replaced with a fresh medium mixture. HUVEC cells proliferation was measured after 5-day cultivation by using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA). The growth of HUVEC in their regular media, in media supplemented with concentrated media from B210 cells, and in media enriched with concentrated media from ES-producing cells was tested in 24, 32 and 32 parallels, respectively.

Animal experiments. Six- to eight-week old Balb/c female mice from Charles Rivers, Germany, were used. All experiments were carried out in accordance with the Guidelines for Animal Experimentation valid in the Czech Republic. Mice were inoculated i.v. with counts of 10^6, $5x10^5$ or 10^5 cells and were followed for at least 75 days. In all experiments the 3rd passage cells derived from the big frozen stocks were used. In the case of gene-modified cells, ES production was always monitored prior to cell administration. In all instances freshly harvested cells were washed three times in PBS before administration. Experimental animals were humanely sacrificed using Narcotan (Zentiva, Czech Republic).

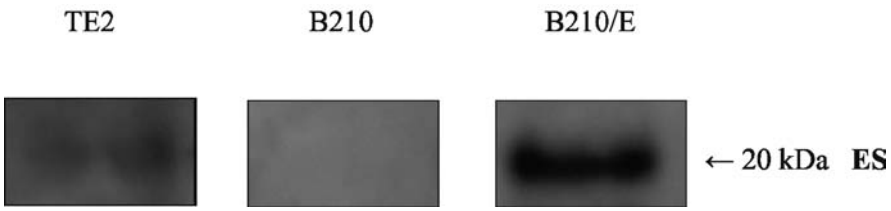

Figure 1. Endostatin (ES) production by B210/E cells. B210 cells were transfected with plasmid carrying the genes for murine endostatin (ES) and blasticidine resistence. The transduced B210/E cells were isolated in the presence of blasticidine. Western blotting with cell lysates and polyclonal goat anti-endostatin antibody. TE2 cells served as a positive control. Parental B210 cells served as a negative control.

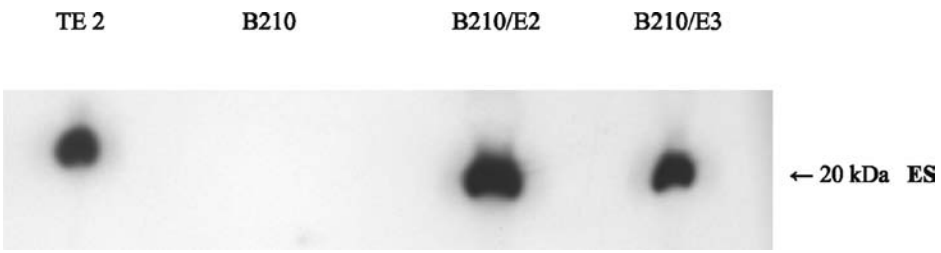

Figure 2. Endostatin (ES) production by transduced B210/E2 and B210/E3 cells. TE2 cells served as a positive control and B210 as a negative control. Western blotting with concentrated media and polyclonal goat anti-endostatin antibody.

Autopsy and histological investigation. Both thoracic and abdominal cavities were inspected. For histological investigation, selected tumors and organs were removed and immediately placed into 10% buffered formaline (in PBS). Using a microtome (Leova) parafin-embedded samples were cut into 3-5-μm sections. Paraffin was removed with ethanol and xylene (Penta, Czech Republic) and the slices were stained with hematoxylin and eosin (Dako, Denmark).

Immunohistochemistry (ES). Deparaffinized sections from tumors and spleens were incubated with goat polyclonal anti-mouse-ES antibody (Sigma-Aldrich) in Dako REAL Antibody Diluent (Dako) (1:250) for 1 h. Samples were washed in PBS and subsequently treated with the Universal Immuno-enzyme Polymer reagent Histofine® (Nichirei Biosciences, Tokyo, Japan), following the manufacturer's instructions. Sections untreated with anti-ES antibody served as controls. A further set of controls was represented by sections from organs taken from B210-inoculated animals. DAB+ substrate chromogen (Dako) was used for visualization. Finally, cells were stained with hematoxylin and eosin (Dako). Materials taken from mice inoculated with the original B210 cells were used as a negative control.

FISH. After deparaffinization, the slices were treated with sodium thiocyanate (Sigma-Aldrich), digested by protease II (3 mg in 40 ml of physiological saline, pH ≤2.0) (Abbott Molecular, IL, USA) for 75 min and fixed in bufferred formaline (solution in PBS). An LSI BCR/ABL Dual Color, Dual Fusion Translocation Probe (Abbott Molecular) was used for the detection of the *BCR-ABL* fusion gene, following the manufacturer's instructions. Fluorescence signals were detected with the use of an Olympus ProVis XC70 microscope.

Statistical methods. Statistical data were analysed using Prism Software Version 5.0 (Graph-Pad Software, San Diego, CA).

Results

After transfection with the plasmid carrying the genes for mouse ES and for resistance to blasticidine, cells were cultivated in the presence of blasticidine, in parallel with mock-transfected cells. Transduced cells were readily selected and grew well in this medium, while control nontransfected cells died out within 10 days. The lysates of cells growing in the presence of blasticidine were tested for the production of ES. The results of WB shown in Fig. 1 indicate that ES was produced by these cells. From the population of the transduced cells we isolated 10 cell clones. Concentrated media from their cultures were subsequently tested for ES production using WB. Six of these clones produced ES in amounts detectable by WB. For subsequent tests we selected two of these clones, namely that one with the highest (clone B210/E2) and one with a lower production of ES (clone B210/E3) Fig. 2. The production of ES remained stable in the course of 15 *in vitro* passages (data not shown).

To demonstrate that the ES produced by the transduced cells was biologically active, its influence on the proliferation of HUVEC cells was tested. Media from the parental B210 culture and from the B210/E2 culture were used in this assay. The optical density of cells treated with medium M200 was taken as 100%. The results shown in Fig. 3 indicate that the medium from ES-producing cells, but not from the parental, unmodified B210 cells, reduced the proliferation of HUVEC cells ($p<0.001$).

Next, we tested the production of the $p210^{bcr\text{-}abl}$ protein by the gene-modified cells. The results are shown in Fig. 4. They suggested that production of the $p210^{bcr\text{-}abl}$ protein was somewhat higher in the transduced cells than in the parental B210 cells. This result was confirmed in repeated tests.

To further characterize these cells we tested them for the expression of MHC I molecules. As indicated in Fig. 5, neither the original B210 cells nor their gene modified sublines produced

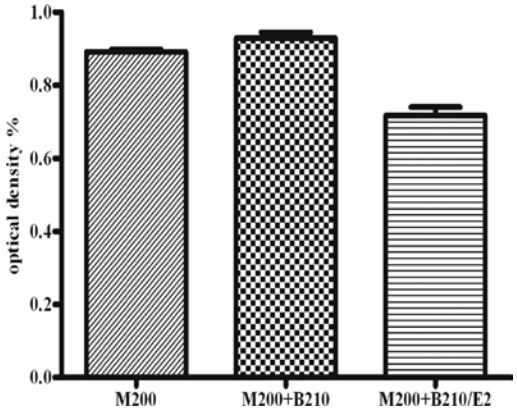

Figure 3. HUVEC proliferation assay *in vitro*. HUVEC cells cultivated in media M200, in media M200 supplemented with concentrated RPMI medium from parental B210 cells (M200+B210) and from endostatin-producing B210/E2 cells (M200+B210/E2).

detectable amounts of MHC I molecules when cultivated *in vitro*, this indicating that the down-regulation of the MHC I present in B210 cells was not altered by the genetic manipulation performed.

Since we had previously demonstrated that the production of ES in another mouse cell line was associated with the loss of oncogenicity connected with the suppression of the IL1α production, we tested both the B210 cells and their ES producing sublines for the presence of this cytokine. The production of IL-1α was not detected in any of these cells (data not shown).

To determine the oncogenic potential of the ES-producing cells, mice were inoculated intravenously with the parental and the ES-producing cells. The results obtained in animals inoculated with 10^6 cells are shown in Fig. 6. As expected, all mice inoculated with the parental B210 cells died of leukemia before day 20. The survival of animals inoculated with the ES

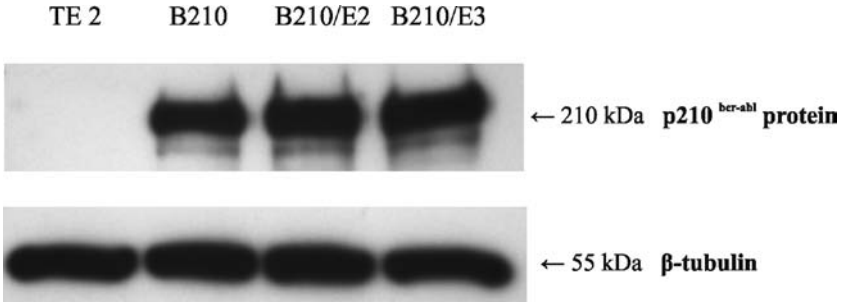

Figure 4. Production of $p210^{bcr\text{-}abl}$ protein by parental B210 cells and by B210/E2 and B210/B3. Western blotting with cell lysates and monoclonal abl-antibody. TE-2 cells served as negative control. β-tubulin served as protein loading control.

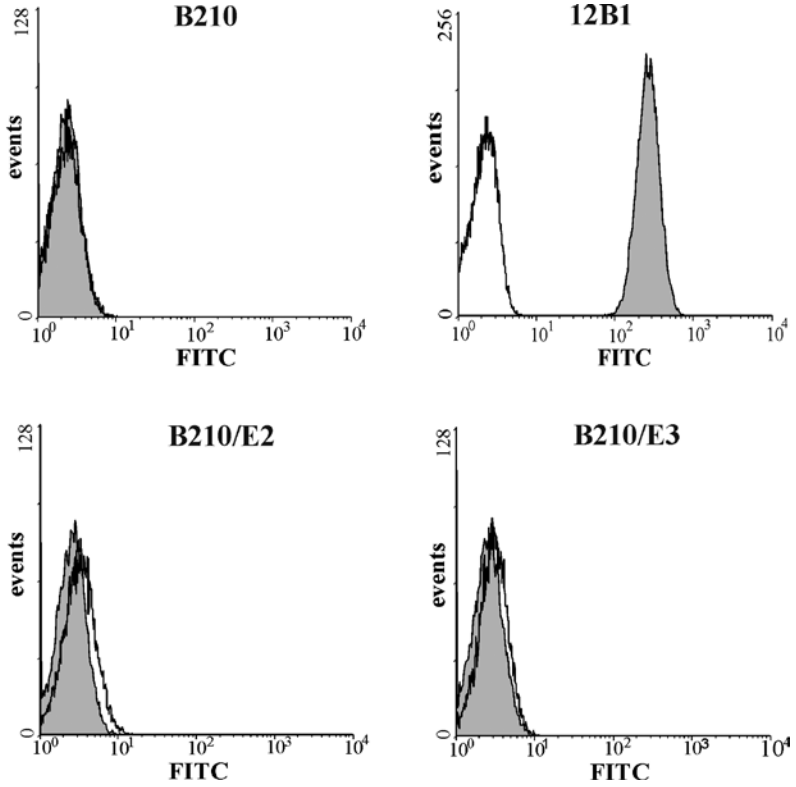

Figure 5. Expression of MHC class I molecules on parental B210 cells and on B210/E2 and B210/E3 cells: empty histograms represent cells incubated with isotype control antibodies; filled histograms represent cells incubated with specific antibodies. B210 cells served as a negative control, 12B1 cells served as a positive control.

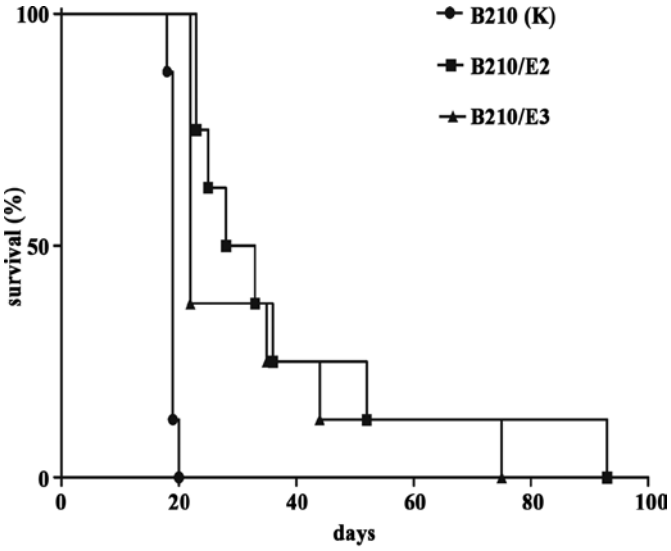

Figure 6. Oncogenicity of parental B210 cells and B210/E2 and B210/E3 cells; cells (10^6) were inoculated intravenously. Eight animals per group were used.

Table I. Incidence of solid tumors in mice inoculated intravenously with parental B210 cells and B210/E2 and B210/E3 cells.

No. of cells inoculated	B210	B210/E2	B210/E3
10^6	0/8[a]	4/8	3/8

[a]No. of animals with solid tumor/no. of animals inoculated.

producing cells was prolonged ($p<0.001$). Surprisingly, a number of mice, which did not succumb to leukemia in the course of a 30-day period after inoculation, later on developed multiple solid intraabdominal, intrathoracic and subcutaneous tumors. As shown in Table I, solid tumors were observed in nearly half of the animals inoculated with 10^6 gene-modified cells. A solid tumor was also seen in 1 of 8 animals inoculated with $5x10^5$ B210/E2 cells, but such tumors were never detected in animals inoculated with 10^5 cells (data not shown). A typical intraabdominal tumor induced by B210/E2 cells is shown in Fig. 7A. These tumors were rapidly growing enveloping different organs, however, mucous membranes were never afflicted (Fig. 7B). Histologically, the tumors were diffuse lymphomas composed of lowly differentiated cells with irregular nuclei and roughly granular chromatin. They were well vascularized (Fig. 7C). Tumors induced by the two transduced cell lines somewhat differed. B210/E2 tumors grew more aggressively and tended to spread more rapidly. B210/E3 induced tumors were smaller, less diffuse and were well separated from the adherent tissues. Using FISH, we were able to detect the *BCR-ABL* fusion gene in the tumor cells (Fig. 7D), this proving that the tumors had been induced by the cells inoculated. In addition, using polyclonal anti-ES antibody, we demonstrated ES production in the tumor cells (Fig. 7E). From several tumors we isolated cell lines producing ES demonstrable in cell lysates by WB. These findings convincingly showed that the solid tumor formation was not due to the loss or silencing of the ES gene.

In autopsied solid-tumor bearing animals, hepatosplenomegaly similar to that seen in animals, which had succumbed to leukemia, was also observed. Apparently, it was associated with the infiltration by leukemic cells. Leukemic cells were demonstrated in blood smears (data not shown) and also in spleens as revealed by both the hybridization test (Fig. 8A) and immunohistochemistry (Fig. 8B).

Discussion

In the present series of experiments we used B210 cells, a mouse cell line transformed by the *BCR-ABL* fusion gene, which are capable of inducing leukemia-like disease after intravenous inoculation into syngeneic animals. For the isolation of ES-transduced cells, we used the same plasmid, i.e., a plasmid carrying the mouse ES gene and the gene for blasticidine resistance, as in previous experiments (10) and the transduced cells were isolated in medium supplemented with blasticidine. The clonal analysis revealed that the population was quite heterogeneous as far as the production of ES is concerned. For further experiments two clones were selected, one with high and one with a relatively low production of ES, to monitor the possible influence of the quantity of the ES produced on the behavior of the cells. Prior to performing *in vivo* experiments, we tested the two lines *in vitro*. As expected, the transduced cells mimicked the parental cells by the downregulation of MHC class I molecules and also by the expression of other surface markers tested (Petrackova *et al*, unpublished data). Trying to find an analogy with the system we had studied previously, we also monitored the expression of IL1α. These efforts failed completely. We did not find detectable amounts of this cytokine either in the parental cells or in the transduced cells.

Quite surprising were the results obtained when we tested the oncogenic potential of the ES-producing cells. When compared with the parental cells, the transduced cells were partially attenuated. While all mice inoculated with 10^6 parental B210 cells succumbed to leukemia before day 20 after inoculation, all animals inoculated with the same dose of the transduced cells survived this critical period. However, after day 30 they started to develop multiple rapidly growing intrathoracic, intraabdominal and subcutaneous solid tumors. Their cells produced ES *in situ* and cell lines derived from them also produced ES, this proving beyond reasonable doubt that the capability of the transduced cells to induce solid tumors was not due to the loss or silencing of the transgene. In spite of the production of ES, the tumors were well vascularized without any detectable signs of necrosis. Thus, it seems clear that ES production was insufficient to suppress angiogenesis in the present system. The mechanisms of its failure are not understood at this moment. Still, several explanations may be offered. The activities of pro-angiogenic molecules present in the tumor environment or produced by the tumor cells themselves may have been able to sustain the aberrant angiogenesis. It should be recalled that increased expression of pro-angiogenic factors in *BCR-ABL*-transformed cells has been reported (18), thus these cells might be well equipped to resist the effects of ES or similar substances. It has also been reported that the growth of tumors induced by cells gene-modified to express ES or other endogenous angiogenic inhibitors, such as thrombospondin 1 or tumstatin, was associated with an overexpression of several pro-angiogenic factors such as VEGF or PDGF; this

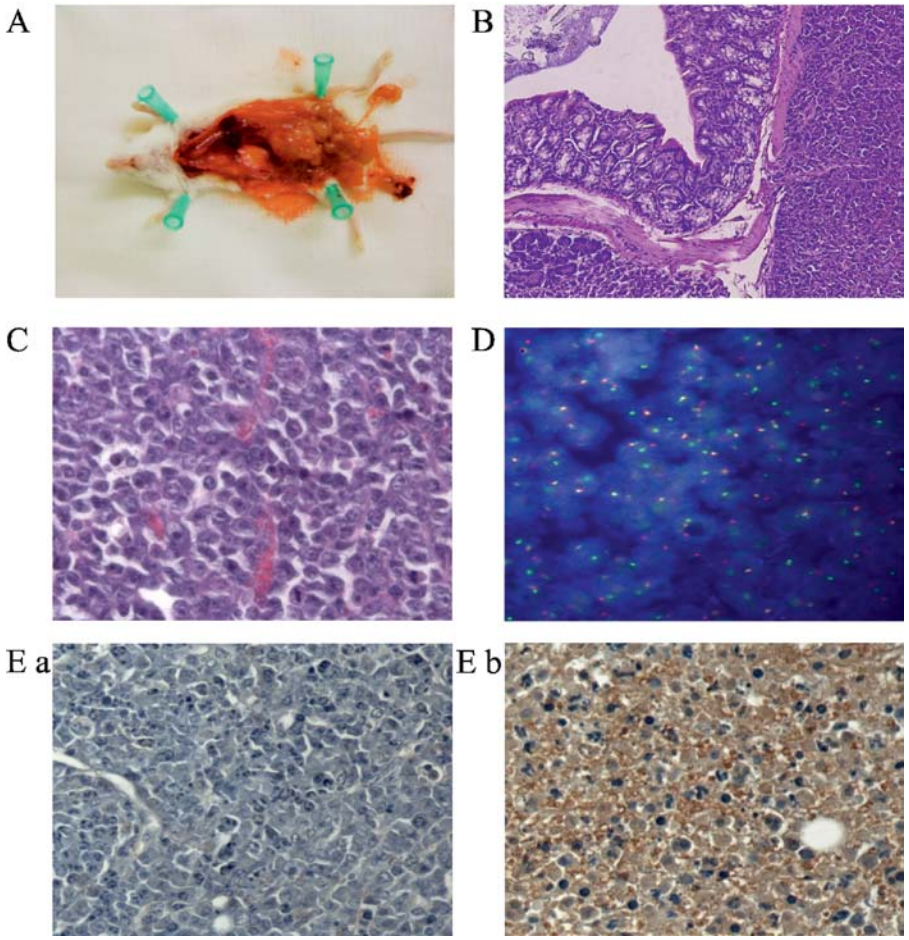

Figure 7. Intraabdominal tumor in a mouse inoculated intravenously with 10^6 B210/E2 cells. Autopsy carried out on day 52 after inoculation (A). Tumor enveloping intestine induced after intravenous inoculation of 10^6 B210/E2 cells. Stained with hematoxylin and eosin (x100) (B). Well-vascularized tumor induced after intravenous inoculation of 10^6 B210/E2 cells. Stained with hematoxylin and eosin (x400) (C). Presence of BCR-ABL fusion gene (orange fluorescent spots) in tumor induced after intravenous inoculation of 10^6 B210/E2 cells, as determined by FISH (x1000) (D). Determination of endostatin production in tumor induced by B210/E2 by means of polyclonal goat anti-endostatin antibody and Histofine (E). (E) (a), Untreated with anti-endostatin antibody, treated with Histofine. (b) Treated with anti-endostatin antibody and Histofine (x200).

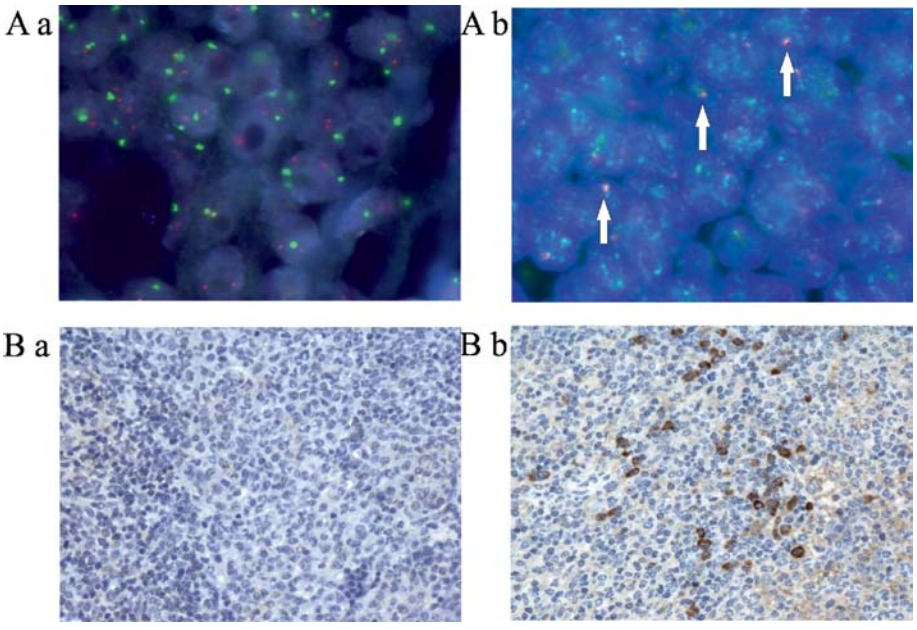

Figure 8. Presence of BCR-ABL-positive cells (orange fluorescent spots, arrows) in spleen of a mouse inoculated with B210/E2 cells, as determined by FISH (A). (A) (a), Spleen from a healthy mouse. (b), Spleen from a mouse with tumor induced by B210/E2 cells (x1000). Presence of endostatin-producing cells in spleen of a mouse with tumor induced by B210/E2 cells, as determined by polyclonal goat anti-endostatin antibody and Histofine (B). (B) (a), Spleen from a healthy mouse. (b), Spleen from a mouse with tumor induced by B210/E2 cells (x200).

was not seen in tumors formed by the parental gene-unmodified cells (19). It has also been shown that pharmacologic inhibitors targeting the VEGF pathway can alter the tumor biology, viz. heighten its invasiveness and increase its metastatic potencial, possibly as an adaptation reaction leading to activation of growth programs different from those used in normal tumor growth (20,21). It is, however, also possible that the vasculature network in the present system was composed of dedifferentiated tumor cells which transdifferentiated into cells expressing endothelial markers. This phenomenon known as vasculogenic mimicry (22,23) has been reported to be resistant to endostatin (24).

Another observation that might be of interest concerns the differences in the *in vivo* behavior of the tumors induced by the high and low ES-producers, although no marked differences were observed in their histological patterns. However, it is hard to assume that the differences found were associated with the extent of ES production. It is more likely that they just reflected an interclonal variation. Certainly, more than two clones differing in ES production should be tested prior to making even a cautious suggestion that such an association might exist.

It is difficult to explain why the ES-producing cells tended to form solid tumors, which are extremely rare in animals inoculated with their parental cells. Even the solid tumor-bearing animals had signs of leukemia, as evidenced by hepatosplenomegaly and the presence of tumor cells in peripheral blood smears and in their spleens. It is possible to speculate that the original B210 cells have an inherent capability of inducing solid tumors as the transduced cells did, but that it cannot manifest itself because the incubation period for solid tumor formation is quite long and the animals inoculated with the parental cells die of leukemia before they can develop such tumors. In this respect it may be of interest that in the recent past we observed an increased occurrence of solid tumors in mice inoculated with B210 cells and then treated for some time with imatininib mesylate (IM). Some of these animals, which did not succumb to leukemia developed lymphoma-like tumors weeks after IM treatment had been stopped. Some solid tumors also developed in few animals immunized with plasmids carrying the *BCR-ABL* fusion gene (unpublished data). Whenever in the previous experiments solid tumors appeared they were always detected 40 days or more after the inoculation of the cells. Should this reasoning be correct then one could conclude that in the present experiments ES production resulted in an attenuation of leukemia-like disease but did not significantly influence the capability of B210 cells to induce solid tumor formation. Experiments aiming at a more detailed analysis of the phenomena observed are under preparation.

Acknowledgements

We thank Professor K. Smetana and Dr S. Lisova for valuable comments on some histological findings. This work was supported by MZCR IGA grant no. NS 10634-3/2009 and by MZ0UHKT 2005.

References

1. Li WW, Hutnik M and Gehr G: Antiangiogenesis in haematological malignancies. Br J Haematol 143: 622-631, 2008.
2. Folkman J: Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat Med 1: 27-31, 1995.
3. Kaban K and Herbst RS: Angiogenesis as a target for cancer therapy. Hematol Oncol Clin North Am 16: 1125-1171, 2002.
4. Cao YH: Tumor angiogenesis and molecular targets for therapy. Front Biosci 14: 3962-3973, 2009.
5. Herbst RS, Mullani NA, Davis DW, *et al*: Development of biologic markers of response and assessment of antiangiogenic activity in a clinical trial of human recombinant endostatin. J Clin Oncol 20: 3804-3814, 2002.
6. Faye C, Chautard E, Olsen BR and Ricard-Blum S: The first draft of the endostatin interaction network. J Biol Chem 284: 22041-22047, 2009.
7. Yoon SS, Eto H, Lin CM, Nakamura H, Pawlik TM, Song SU and Tanabe KK: Mouse endostatin inhibits the formation of lung and liver metastases. Cancer Res 59: 6251-6256, 1999.
8. Sauter BV, Martinet O, Zhang WJ, Mandeli J and Woo SLC: Adenovirus-mediated gene transfer of endostatin in vivo results in high level of transgene expression and inhibition of tumor growth and metastases. Proc Natl Acad Sci USA 97: 4802-4807, 2000.
9. Folkman J: Antiangiogenesis in cancer therapy-endostatin and its mechanisms of action. Exp Cell Res 312: 594-607, 2006.
10. Lakatosova-Andelova M, Duskova M, Lucansky V, Paral P and Vonka V: Effects of endostatin production on oncogenicity and metastatic activity of HPV16-transformed mouse cells: role of interleukin 1 alpha. Int J Oncol 35: 213-222, 2009.
11. Vonka V: Immunotherapy of chronic myeloid leukemia: present state and future prospects. Immunotherapy 2: 227-241, 2010.
12. Lucansky V, Sobotkova E, Tachezy R, Duskova M and Vonka V: DNA vaccination against bcr-abl-positive cells in mice. Int J Oncol 35: 941-951, 2009.
13. Petrackova M, Sobotkova E, Duskova M, Jinoch P and Vonka V: Isolation and properties of gene-modified mouse bcr-abl-transformed cells expressing various immunostimulatory factors. Neoplasma 56: 194-201, 2009.
14. Sobotkova E, Ludvikova V, Petrackova A, *et al*: Characteristic of two mouse bcr-abl-transformed cell lines: I. General properties of the cells. Folia Biologica 51: 12-18, 2005.
15. Daley GQ and Baltimore D: Transformation of an interleukin-3-dependent hematopoietic-cell line by the chronic myelogenous leukemia-specific P210Ber/Abl protein. Proc Natl Acad Sci USA 85: 9312-9316, 1988.
16. McLaughlin J, Chianese E and Witte ON: In vitro transformation of immature hematopoietic cells by the P210 BCR/ABL oncogene product of the Philadelphia chromosome. Proc Natl Acad Sci USA 84: 6558-6562, 1987.
17. Jelinek F, Sobotkova E and Vonka V: Characteristics of two mouse bcr-abl-transformed cell lines. II. Pathological lesions induced in mice. Folia Biologica 51: 93-102, 2005.
18. Sillaber C, Mayerhofer M, Aichberger KJ, Krauth MT and Valent P: Expression of angiogenic factors in chronic myeloid leukaemia: role of the bcr/abl oncogene, biochemical mechanisms, and potential clinical implications. Eur J Clin Invest 34: 2-11, 2004.
19. Fernando NT, Koch M, Rothrock C, Gollogly LK, D'Amore PA, Ryeom S and Yoon SS: Tumor escape from endogenous, extracellular matrix-associated angiogenesis inhibitors by up-regulation of multiple proangiogenic factors. Clin Cancer Res 14: 1529-1539, 2008.
20. Ebos JML, Lee CR, Cruz-Munoz W, Bjarnason GA, Christensen JG and Kerbel RS: Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis. Cancer Cell 15: 232-239, 2009.
21. Paez-Ribes M, Allen E, Hudock J, *et al*: Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. Cancer Cell 15: 220-231, 2009.
22. Maniotis AJ, Folberg R, Hess A, *et al*: Vascular channel formation by human melanoma cells in vivo and in vitro: Vasculogenic mimicry. Am J Pathol 155: 739-752, 1999.
23. Hillen F and Griffioen AW: Tumour vascularization: sprouting angiogenesis and beyond. Cancer Metastasis Rev 26: 489-502, 2007.
24. Van der Schaft DWJ, Seftor REB, Seftor EA, *et al*: Effects of angiogenesis inhibitors on vascular network formation by human endothelial and melanoma cells. J Natl Cancer Inst 96: 1473-1477, 2004.