Stromal Expression of c-Ets1 Transcription Factor Correlates with Tumor Invasion

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ABSTRACT

The stroma reaction has an important role in tumor growth, invasion, and metastasis. In various invasive human carcinomas, as well as in a mouse model for tumor invasion, transcripts encoding the transcription factor c-Ets1 were detected within stromal fibroblasts, whereas they were absent in epithelial tumor cells. This expression of c-Ets1 was often increased in fibroblasts directly adjacent to neoplastic cells. Endothelial cells of stromal capillaries were also positive for c-Ets1 expression. In contrast, fibroblasts of corresponding noninvasive lesions and of normal tissues were consistently negative. In cultured human fibroblasts stimulated by basic fibroblast growth factor and tumor necrosis factor, the expression of c-Ets1 correlated with the accumulation of transcripts for potential target genes, collagenase-1 and stromelysin-1. The same correlation was observed in some of the invasive carcinomas investigated. These results suggest that c-Ets1 participates in the regulation of tumor invasion in vivo.

INTRODUCTION

The c-ets1 proto-oncogene has been identified as the cellular progenitor of the viral oncogene v-ets that is associated with v-myb in the genome of the avian leukemia retrovirus E26 (1, 2). The Ets1 proteins are very conserved in species ranging from Drosophila to human (3); they are the founders of a new family of transcriptional regulators (reviewed in Refs. 4 and 5). The proteins belonging to this “Ets family” share a sequence of about 85 amino acids, the DNA binding domain that recognizes target DNA sequences containing a central GGAAT core motif (6—9). Although little is known about the cellular genes regulated by c-Ets1 in vivo, it appears that c-Ets1 can either contribute to positive transcriptional activation, as shown on the long terminal repeat of the Moloney murine sarcoma virus (10), or to negative regulation, as shown by its repressive activity on the T-cell receptor-β enhancer (11). In addition, the activity of c-Ets1 can be modulated by its interaction with other transcription factors; c-Ets1 cooperates with the Fos/c-Jun complex, AP-1, for transcriptional activation of the polyoma virus enhancer (12), and it activates the HTLV1 long terminal repeat synergistically with SP1 (13).

Initial studies in hatched chicken (14, 15) and in mouse tissues (16) have shown that c-ets1 mRNAs are preferentially detected in lymphoid organs. However, in situ analysis of developing embryos revealed a broader expression pattern. In chick (17) and mouse (18, 19) embryos, c-ets1 mRNAs are also expressed in mesenchymal cells adjacent to epithelial structures, where inductive epithelio-mesenchymal interactions occur (19, 20). This expression has been described in the dermis before the formation of epithelial cutaneous structures (21), in the limb bud, in the mesenchyme surrounding the gizzard and the gut, and during branching morphogenesis in the kidney and in the lung near growing and invading epithelial buds (19, 20). c-ets1 transcripts are also abundant during neural crest migration and new blood vessel formation (17, 18), which are physiological examples of tissue invasion.

In malignant tumors, invasion is one of the crucial steps in the metastatic cascade in which changes in cell adhesion and extensive remodeling of the extracellular matrix occur (22). Matrix degrading proteases such as u-PA, collagenase-1, and stromelysin-1 are involved in this process (23). Although their biological activity is thought to be highly regulated at a posttranslational level (Ref. 24; reviewed in Ref. 25), several evidences indicate also a regulation at the transcriptional level (26—29). Transcription factors that take part in the induction of protease genes include AP-1 and c-ets1; in transient cotransfection assays, c-ets1 overexpression leads to transactivation of the stromelysin-1 promoter (7) and might contribute to transcriptional activation of the collagenase-1 gene (32). In addition, a regulatory element containing juxtaposed binding sites for Ets and AP-1 proteins in the human u-PA promoter has been shown to be crucial for its transcriptional activity in different cell lines (33).

In this work, we examined the expression patterns of c-ets1 mRNAs in various human carcinomas and compared them with corresponding benign tumors and other noninvasive lesions. We also investigated c-ets1 expression in a mouse model for tumor invasion and in cultured human foreskin fibroblasts. In parallel to c-ets1, we investigated the expression of u-PA, collagenase-1, and stromelysin-1 mRNAs, both in vitro and in vivo. Our results suggest that c-ets1 participates in the regulation of tumor invasion, presumably by controlling the transcriptional activation of matrix-degrading proteases genes in stromal fibroblasts.

MATERIALS AND METHODS

Tissues. The human carcinomas investigated in this study comprised two squamous cell and one adenocarcinoma of the lung, two ductal breast cancers from females, two adenocarcinomas of the colon, one ductal adenocarcinoma of the pancreas, one papillary thyroid carcinoma, and one glandular prostate cancer (grade 3, according to Ref. 34). For comparison, we examined several benign and noninvasive tumors (four adenomas of the colon without significant dysplasias, one fibroadenoma of the breast, one benign cystadenoma of the ovary, and one of the borderline-type), nontumor-associated fibrotic lesions (one fibrocystic breast disease presenting focal epithelial proliferations without nuclear atypia and two arterio-sclerotic intimal plaques from coronary arteries), and normal tissues from colon, lung, breast, and prostate. All samples were from adults (ages 39 to 74 years).

To produce the experimental mouse model, 1 mm3 pieces of a human undifferentiated ovarian carcinoma from a 59-year-old Caucasian woman were s.c. transplanted into SCID mice (“Cb 17,” generously provided by J. Y. Cesbron, Institut Pasteur de Lille, France). The tumors were allowed to grow to approximately 1 cm3 before the animals were sacrificed; then the tumors were excised with the surrounding tissues.

The abbreviations used are: u-PA, urokinase-type plasminogen activator; SCID, severe combined immunodeficiency; cDNA, complementary DNA; PAI-1, plasminogen activator inhibitor type 1; TNFα, tumor necrosis factor α; TGFβ, transforming growth factor β; PDGF, platelet-derived growth factor; bFGF, basic fibroblast growth factor; PMA, phorbol 12-myristate 13-acetate.
Probes. RNA probes for in situ hybridization and nick-translated probes for Northern blot analysis were prepared from the following cDNA fragments: a 825-base pair fragment of human c-etsl (nucleotides 260–1086, as described in Ref. 35) cloned into pSP64 and pSP65 (Promega) that does not cross-hybridize with c-ets-2 mRNAs (36); the 1.6-kilobase SacI-KpnI fragment of mouse c-etsl cDNA (37) cloned into Bluescript SK (Stratagene); these human and mouse probes gave species-specific hybridization signals; a fragment of human u-PA described by Pyke et al. (27) and cloned into Bluescript KS (Stratagene) for in situ hybridization and the 600-base pair, EcoRI-PstI fragment of the human u-PA cDNA for Northern blot analysis; the HindIII-PstI fragment of the bovine PAI-i cDNA; the complete cDNA of human collagenase-1 (30) cloned into pSP64 and pSP65 (Promega) that does not cross-hybridize with c-etsl cDNA and the 1.6-kilobase XbaI fragment of the human stromelysin-1 cDNA; and a 301-base pair fragment of the human stromelysin-1 cDNA cloned into pGEM 3 (Promega; Biotech), which extends 221 base pairs into the 3' untranslated region and is specific for stromelysin-1 versus stromelysin-2 and stromelysin-3 (38).

In Situ Hybridization. The detailed procedures for fixation, embedding of the tissue samples, and in situ hybridization were as described previously (27, 39). Both 35S-labeled antisense and control sense RNA probes were used, and the sense probes gave negative results on all tissue sections examined. In order to compare the localization of positive signals for c-etsl mRNAs on one hand and for the three proteases transcripts on the other, hybridizations with the different probes were carried out on neighboring sections. We used May-Grunwald-Giemsa, hematoxylin and eosin, or fluorescent (Hoechst 33258) counterstaining, depending on the intensity of the signals. The c-etsl hybridization signals were sometimes low, and they were in these cases most easily visualized by dark-field illumination of sections after fluorescent counterstain. Under these circumstances, hybridizations and hematoxylin and eosin staining were carried out on different sections.

RESULTS

Expression of c-etsl within the Stromal Fibroblasts of Human Carcinomas. Several invasive human carcinomas from lung, breast, colon, pancreas, thyroid, and prostate (Table 1) were examined. They revealed usual histological features and had induced, with the exception of the prostate cancer, a typically developed stromal component composed of varying amounts of fibroblasts with collagen fibers, new blood vessels, and inflammatory cells, which were mainly lymphocytes. As usually observed (41), the prostate cancer invaded the preexisting fibromuscular stroma without a remarkable stromal component.

In situ hybridization revealed positive signals for c-etsl within the fibroblasts of all carcinomas which had induced a fibrous stroma (Table 1; Fig. 1). The pancreatic carcinoma was the only tumor where positive fibroblasts formed only a few clusters within the stroma. The preexisting fibromuscular prostate stroma was negative for c-etsl expression. The c-etsl transcripts were frequently found within the fibroblasts directly adjacent to the invasive tumor formations, whereas no transcripts were detected in more distant fibroblasts (Fig. 1, A and B) and in fibroblasts of neighboring mature normal connective tissues. As previously reported in well vascularized human tumors (36), we found an expression of c-etsl within the endothelial cells of stromal capillaries and within stromal lymphocytes (data not shown). In contrast, mature blood vessels of larger size were always negative. The epithelial tumor cells in all cases studied never presented positive signals for the c-etsl antisense probe.

Expression of c-etsl within the Fibrous Stromata of Noninvasive Tissues. In order to compare the transcription of c-etsl between invasive and noninvasive tissues, we examined several benign and noninfiltrating tumors (colon adenomas, a fibroadenoma of the breast, and two cystadenofibromas of the ovary), nontumor-associated fibrotic lesions as fibrocystic breast disease, and arteriosclerosis as well as samples from normal lung, breast, colon, and prostate. The stromal component of these tissues revealed usual histological features, with the presence of varying numbers of fibroblasts. As usually seen, the cellular stroma of the ovarian cystadenofibromas resembled the normal ovarian stroma; the borderline tumor (Fig. 2C) showed atypical papillary epithelial proliferations without invasion.

Without exception, all of these tissues were negative for c-etsl expression, both within their fibrous or fibromuscular stroma and within their epithelial constituents (Fig. 2). As previously reported (36), normal lymphocytes were positive.

Expression of c-etsl within SCID Mice after Transplantation of a Human Tumor. To evaluate whether human tumor cells could trigger c-etsl expression in another host, we investigated the stroma

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<th>Tumor cells</th>
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<td>Ductal cell adenocarcinoma of the pancreas</td>
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<td>Papillary thyroid carcinoma</td>
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<td>Prostatic adenocarcinoma</td>
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* Semiquantitative estimation of positive cells: +, <10%; ++, 10-50%; ++++, >50%; -, no signal; ND, not determined; coil 1, collagenase-1; strom 1, stromelysin-1.

a Preexistent fibromuscular prostate stroma.
reaction of SCID mice to a s.c. implanted human ovarian carcinoma. The tumor was poorly differentiated and locally invaded the mouse tissues. It had induced in the host a pronounced stroma reaction analogous to that found in human carcinomas. With a mouse-specific c-etsl probe, positive signals were observed within stromal fibroblasts and capillaries (Fig. 3), whereas no positive signals were detected with the human c-etsl probe (data not shown), which proved the mouse origin of the tumor stroma. As for the human carcinomas described above, the expression within stromal fibroblasts could also be found directly adjacent to the invasive tumor front (Fig. 3B). No hybridization signals were detected for either probe on normal fibrous tissues and blood vessels of the mouse-host or on the human tumor cells themselves.

**Fig. 1.** Expression of c-etsl within the fibroblastic stroma of invasive human tumors. A and B, poorly differentiated human colon adenocarcinoma. Positive signals were seen directly adjacent to the invasive formations. Dark-field (A) and bright-field (B) illumination of the same section counterstained with May-Grünwald-Giemsa. C and D, well-differentiated glandular breast carcinoma. C, dark-field illumination of a section counterstained with Hoechst 33258. D, neighboring section counterstained with hematoxylin and eosin showing tumor morphology with a fibroblast rich stroma. Bar, 100 µm.

**Fig. 2.** Absence of c-etsl expression in a normal colon mucosa (A), in a fibroadenoma of the breast of the so-called intracanalicular type (B), and in a proliferating cystadenofibroma of the ovary of the borderline type (C). Dark-field illumination after fluorescent counterstaining of the nuclei. Bar, 100 µm.

**Fig. 3.** Expression of c-etsl in the reactive stroma after transplantation of a poorly differentiated human tumor into a SCID mouse. A, positive signals for mouse c-etsl mRNAs within the fibroblastic stroma of inner parts of the tumor. B, mouse c-etsl transcripts within the stromal fibroblasts directly adjacent to the front of invasion, as demonstrated by the mouse antisense probe. C, hematoxylin and eosin counterstained neighboring section of B. A and B were obtained by dark-field illumination of sections counterstained with Hoechst 33258. The human c-etsl antisense probe gave negative results. Bar, 100 µm.

**c-etsl, Collagenase-1, Stromelysin-1, and u-PA Gene Expression in Cultured Human Fibroblasts in Response to Cytokines or PMA.** We subsequently investigated whether cytokines, which might be secreted by tumor cells, activated macrophages, or mast cells in the tumor stroma (42–44) could induce c-etsl expression in cultured fibroblasts. In a first series of experiments, we found that conditioned media (see “Materials and Methods”) of various tumor-derived epithelial cell lines, HeLa, Colo 320, and MCF7 cells, induced c-etsl expression in subconfluent human foreskin fibroblasts (data not shown). Using purified cytokines, we found that bFGF and TNFα increased the amount of c-etsl mRNAs (Fig. 4) and proteins, whereas TGFβ1 or PDGF had only little or no effect (Fig. 4).

Since cotransfection experiments have shown that c-etsl might be involved in the transcriptional regulation of u-PA, collagenase-1, and stromelysin-1 genes (7, 32, 33), we were interested to examine the relationship between c-etsl expression and the transcription of these proteases genes. In human foreskin fibroblasts, the amounts of c-etsl mRNAs induced by bFGF, TNFα, TGFβ1, or PDGF correlated with the levels of collagenase-1 and stromelysin-1 mRNAs (Fig. 4). Only bFGF strongly induced the accumulation of u-PA transcripts. In addition, the phorbol ester PMA, which is known to activate the expression of extracellular matrix-degrading proteases (45, 46), also increased the amount of c-etsl mRNAs as well as the amount of u-PA, collagenase-1, and stromelysin-1 transcripts (Fig. 4). This correlation between the expression of c-etsl and the accumulation of these protease gene transcripts was also observed in human embryonic lung fibroblasts (MRC5 cells; data not shown).

**Correlation between c-etsl, u-PA, Collagenase-1, and Stromelysin-1 Gene Transcription in Invasive Human Carcinomas.** In view of these in vitro correlations, we examined the expression of u-PA, collagenase-1, and stromelysin-1 gene transcripts within human carcinomas in comparison with that of c-etsl. Seven of the nine tumors were positive for at least two of the proteases (Table 1). The transcripts were mainly found, as for c-etsl, within the fibroblastic tumor stroma. In five cases, there were additional focal, positive signals within the tumor cells. On neighboring sections, the two squamous cell carcinomas of the lung showed, within the stromal

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4 F. Gilles, manuscript in preparation.
fibroblasts, a topographical colocalization of the positive signals for c-etsl and those for collagenase-1 (Fig. 5, compare B and C). In one of these tumors, this colocalization was also observed for u-PA transcripts (data not shown). In the glandular lung carcinoma, a topographical coexpression for c-etsl and stromelysin-1 could be seen within the fibroblastic stroma (Fig. 5, compare E and F). In the other tumors that expressed protease transcripts in stromal fibroblasts, a superposition with the positive signals for c-etsl was found in restricted areas. Endothelial cells and stromal lymphocytes were negative for all three proteases. The prostate cancer was negative within the preexistent fibromuscular stroma for all three enzymes, as it was for c-etsl (data not shown). The noninvasive lesions and normal tissues have been investigated for collagenase-1 and stromelysin-1 expression; they were, without exception, negative (data not shown).

DISCUSSION

In the present study, we have detected c-etsl transcripts within the stromal fibroblasts of different invasive human carcinomas. Conversely, fibroblasts were negative in the stroma of corresponding noninvasive tumors, nontumor-associated fibrotic lesions, and normal tissues. Epithelial tumor cells never expressed c-etsl. The features of c-etsl expression in human carcinomas are reminiscent of the pattern observed in chicken and mouse embryos (17–19), where c-etsl transcripts accumulate in the mesenchyme surrounding growing and invading epithelial buds (19, 20). As for neoplastic epithelia, embryonic epithelia are negative for c-etsl (17). Thus, c-etsl mRNAs are expressed during the course of epithelio-mesenchymal interactions, occurring both during embryonic development or in the stroma reaction to invasive tumors. In this respect, the prostate carcinoma investigated here constitutes an interesting exception. The preexisting fibromuscular prostate stroma invaded by the tumor showed negative for c-etsl. This absence of a remarkable stromal reaction is usually seen (41, 47) but poorly understood.

The fact that in vivo positive signals for c-etsl are often seen within fibroblasts adjacent to the invasive tumor formations suggests that c-etsl transcription might be triggered directly or indirectly by diffusible factors released from the tumor cells. Our in vitro studies using human fibroblasts identify both TNFα and bFGF as possible candidates for such an effect. In favor of this hypothesis, bFGF is commonly expressed by tumor cell lines (43), and TNFα is mainly produced by macrophages activated at the site of neoplastic transformation (42, 44). The fact that both bFGF and TNFα can stimulate the expression of c-etsl mRNAs, whereas TGFβ and PDGF have no effect, suggest that this stimulation does not correlate with fibroblast proliferation since both bFGF and PDGF are mitogenic for these cells. However, whether the in vitro effects of these cytokines on c-etsl expression reflect their activity in vivo remains an open question since the responsiveness of cells to cytokines can be markedly affected by their extracellular matrix environment (48).

The serine protease u-PA and the metalloproteinases collagenase-1 and stromelysin-1 belong to a complex enzymatic cascade that degrades the extracellular matrix and might open the way to invading tumor cells (reviewed in Ref. 22). Cotransfection experiments and mutational analysis of promoter sequences have demonstrated that Ets-binding sites within the promoters of u-PA (33, 49) and collagenase-1 genes (32, 50) are essential for their transcriptional activation and that Ets1 proteins are effective in activating the stromelysin-1 gene promoter (7). In several tumors, the expression of c-etsl superimposed with that of the investigated proteases. In these tumors, it is tempting to speculate that the c-etsl transcription factors take part in the transcriptional control of protease genes expression, either independently or in cooperation with other transcription factors. However, this model does not account for all tumors investigated. In tumors showing positive for c-etsl mRNAs, but in contrast negative for at least one of these proteases, transcription factors cooperating with c-etsl might be absent; alternatively in these tumors, c-Ets1 might be involved in the transcriptional regulation of other genes implicated in tumor invasion, such as the collagenase-4, tissue inhibitor of metalloproteinase-1, or α4-integrin genes that contain essential Ets binding sites in their promoters (51–53). In addition, when protease genes are expressed by tumor cells in which c-etsl is not detected, it is possible that other factors, such as AP1 (33, 54) or other members of the Ets family, take part in their transcriptional regulation. Surprisingly, in the stroma of all tumors investigated, we found endothelial cells to be constantly negative for collagenase-1, stromelysin-1, and uPA, as also reported by others (26, 27). Examination of in vivo angiogenesis models (55) have suggested that angiogenesis requires the degradation of the extracellular matrix, and in vitro studies (46, 56–59) have shown that angiogenic factors can induce the accumulation of u-PA and collagenase-1 transcripts in cultured endothelial cells. Among other possibilities, one may hypothesize that the expression of proteases in endothelial cells has not been detected in vivo because other cell types contribute to extracellular matrix degradation during angiogenesis (reviewed in Ref. 60).

Tumor growth and metastasis appear to involve a variety of interactions between the tumor cells and their surrounding stroma (61, 62). The molecular actors of the stroma reaction characterized thus far are growth factors, matrix-degrading proteases, and adhesion molecules (reviewed in Refs. 22, 25, and 63). To our knowledge, c-Ets1 is the first transcription factor to be detected during the stroma reaction to invasive tumors.

ACKNOWLEDGMENTS

We thank Klaus Remberger (Homburg, RFA) and Bernard Gosselin (Lille, France) for their help in collecting surgical specimens, Ji Hshiong Chen for the gift of the mouse c-etsl probe, Pascale Verde for the human u-PA probe, Michael Pepper for the bovine PAI-1 probe, Peter Angel for the human
collagenase-1 probe, and Susan Quinones for the human stromelysin-1 probe. We thank Jean-Yves Cesbron (Pasteur Institute, Lille) for the generous gift of the SCID mice. We also thank R. Lafaytis, J. H. Chen, and Y. de Launoit for critical reading of this manuscript and Nicole Devassine for patient typing.

REFERENCES


20. Queva, C., Leprince, D., Stéhelin, D., and Vandenbunder, B. p54c-ets-1 and 66k-ets-1, the two transcription factors encoded by the c-ets-1 locus, are differentially expressed during the development of the chick embryo. Oncogene, 8: 2511–2520, 1993.


24. Goldberg, G. I., Marmer, B. L., Grant, G. A., Eisen, A. Z., Wilhelm, S., and He, C. Human 72-kilodalton type IV collagenase forms a complex with a tissue inhibitor of...


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