Simultaneous Suppression of Progression Marker Genes in the Highly Malignant Human Melanoma Cell Line BLM after Transfection with the Adenovirus-5 E1A Gene

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The highly metastatic human melanoma cell line BLM was transfected with the E1A or E1A+E1B regions of adenovirus 5 (Ad5). A series of progression markers, correlated with the malignant phenotype of parental BLM (including calcyclin, thymosin β10, plasminogen activator inhibitors types 1 and 2, urokinase type and tissue type plasminogen activators, vimentin, tissue type transglutaminase, and interleukin-6), was collectively repressed in the transfectants, whereas several control genes were not affected or even induced. The apparently coordinate repression of a set of markers by the same regulator gene, Ad5 E1A in this case, suggests the existence of one pathway under the control of a main switch and predicts that one or more as yet unidentified cellular master genes normally exert this function. A reduced oncogenicity was observed after subcutaneous inoculation of the E1A transfectants into nude mice and provides additional evidence in support of a tumor suppressor function of Ad5 E1A. © 1996 Academic Press, Inc.

Melanocytic tumor progression is thought to evolve through several distinct stages, from normal melanocytes to highly invasive melanoma cells capable of metastasis (1,2). A vast collection of molecular markers associated with subsequent stages of melanocytic tumors and expressed in a number of melanoma derived cell lines (for review, see ref. 3) has been described.

In the past years we have characterized several reverse progression markers, present in non-metastatic human melanoma cell lines, as well as positive progression markers, characteristic for highly metastatic melanoma cell lines (4-10). While changes in gene regulation drive tumor progression, it should be feasible to identify one or a few regulatory genes (e.g. encoding transcription factors or coactivators) that control expression of a set of either reverse or positive progression markers by characterizing the regulatory elements of available marker genes. Alternatively, it may be possible to test a regulatory gene as a candidate to control the expression of a set of related marker genes. The experiments described in this report follow the latter approach, using the human Ad5 E1A gene as a candidate suppressor gene.

While the human Ad5 E1A gene is best known for its transforming properties and related broad influences on gene regulation (for review, see ref. 11), the introduction of Ad5 E1A into highly malignant rodent or human cells induced a reduction of the metastatic potential (reviewed in ref. 12). The reduced metastatic activity in E1A transfected cells correlated with reduced levels of metalloproteinases, e.g. stromelysin (13), and collagenase type I and type

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Abbreviations used are: Ad5, adenovirus type 5; IL-6, interleukin-6; PAI-1, plasminogen activator inhibitor type 1; PAI-2, plasminogen activator inhibitor type 2; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate; tPA, tissue type plasminogen activator; Tgase2, tissue type transglutaminase; uPA, urokinase type plasminogen activator.

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IV (14), or down-regulation of the proto-oncogene neu (15,16). Amplification or overexpression of the normal human c-erbB-2/neu proto-oncogene is a frequent event in many types of human cancers. Renewed ectopic overexpression of neu in E1A transfected ovarian carcinoma cells led to an increased tumorigenicity but hardly re-induced the metastatic phenotype indicating that tumorigenicity and metastasis are related, but separable phenomena (17). Interestingly, E1A activity also stimulated nm23 expression, whose overexpression inhibited metastasis of tumor cells (18). This report describes the effects of ectopic Ad5 E1A expression in the highly metastatic human melanoma cell line BLM, especially the suppression of positive progression marker genes.

MATERIAL AND METHODS

Cell lines and xenografts. Human melanoma cell lines MV3, BLM, 530 and 1F6 (19,20) were grown and xenografts were raised as described earlier (7). Tumor volumes in grafted nude mice were measured weekly and the maximally acceptable tumor size was 15-20 cm³.

Transfection. Transfections were performed in the BLM cell line using lipofectin (BRL, Life Technologies Inc., Grand Island, New York). The BLM cell line was co-transfected with 19 µg of pAd5Xho (E1A+E1B) or pAd5Pst (E1A) (21) and 1 µg of pZipneo (22) carrying the G418 resistance gene, or transfected with pZipneo alone. Stable transfecants were selected in the presence of 1 mg/ml G418 (Gibco Laboratories) in the culture medium. Individual colonies were isolated 2-3 weeks after transfection and selected by immunofluorescence with the M73 anti-E1A mouse monoclonal antibody. After selection, cells were grown in the presence of 0.2 mg/ml G418. As a control the BLM cell line was also treated with lipofectin alone (mock-transfected BLM).

Immunofluorescence. For immunofluorescence studies cells were grown on 12-well multistest slides (ICN Biochemicals Inc., Aurora, Ohio) and subsequently processed following the methanol-acetone fixation protocol (23). The slides were incubated with the M73 anti-E1A monoclonal antibody (24). Slides were washed in PBS (3 X 10 min), and incubated for 60 min with fluorescein isothiocyanate conjugated conjugated immunoglobulins to mouse immunoglobulins (Dakopatts, Glostrup, Denmark). For actin staining, cells were stained for 30 min with rhodamine-phalloidin. After extensive washing with PBS (3 X 10 min), slides were mounted in GELVATOL (Monsanto, AL, USA).

RNA isolation and northern blot analysis. Total RNA was isolated using the lithium-urea procedure as described by Auffray and Rougeon (25). Ten micrograms of total RNA were glyoxylated (26), size fractionated on 1% agarose gels, and blotted onto Hybond N-plus (Amersham, Aylesbury, UK). The hybridization of northern blots was performed according to the method of Church and Gilbert (27) with the addition of 0.1 mg denatured herring sperm DNA per ml of hybridization mixture. To confirm that equal amounts were loaded in each lane, the blots were afterwards hybridized to an 18S ribosomal RNA probe.

DNA probes. The DNA probes for northern blot analysis were as follows; calcyclin (4); thymosin β10 (5); nm23 (9); nm72 (7); Tgase2 (courtesy Dr. P. Davies, Houston, TX; ref. 28); c-src (exon 2; ref. 29); 1.8 kb and 0.45 kb partial cDNA fragments for memA and memB, respectively, two novel human genes with elevated mRNA levels in the highly metastatic human melanoma cell lines MV3 and BLM, and low expression in the non-metastatic cell lines 1F6 and 530 (J. J. M. van Groningen, unpublished results); PAI-1 (30); PAI-2 (31); uPA (30); tPA (31); p53 (courtesy Dr. R. Bernards, Amsterdam, The Netherlands; ref. 32); Ad 5 E1A (21); melanocyte associated antigen ME491 (4,33); heat shock gene hsp70, mouse β-actin, rat vimentin (unpublished). All probes were of human origin unless otherwise indicated.

Cell labeling and immunoprecipitation. Subconfluent cell cultures were labeled for 4 h using 0.5 mCi of [35S]-methionine (Tran35S-Label, ICN; UK) in methionine-free Dulbecco's modified Eagle's medium containing 5% dialyzed fetal calf serum. Lysates were prepared and subjected to immunoprecipitation with M73 anti-E1A monoclonal antibody (24) and analyzed by SDS-PAGE as described elsewhere (8).

IL-6 assay. IL-6 production was assayed by hybridoma growth stimulation assay (34).

RESULTS

Transfection of the E1A or E1A+E1B genes into a highly metastatic cell line. Highly metastatic BLM melanoma cells were co-transfected with E1A+E1B (pAd5Xho) or E1A (pAd5Pst) (21) and pZipneo, or transfected with pZipneo alone. The immunofluorescence test for E1A protein expression yielded one positive E1A transfectant, BLM.E1A, and two positive E1A+E1B transfectants, BLM.E1-1 and BLM.E1-2. These three transfectants were compared with the parental cell line BLM, the mock-transfected parental line using lipofectin only (BLM.mock), and one neo transfectant (BLM.neo). BLM.E1-1 and BLM.E1-2 cell lines had
In cell culture no significant difference was seen in growth rates between the control cell line BLM-1 and BLM-1-2. The various control BLM cells and BLM-1A displayed numerous stress fibres (Fig. 1B). The reverse was true for the transfectants Fx+E/J. This reverse was accompanied by cytoskeletal changes; all cells were stained with rhodamine-phalloidin, with spindle-shaped, epithelioid-like a hA. The morphology of BLM-1A was epithelioid-like, and BLAbenceous revertant fibroblasts. (Fig. 1A, F). The morphology of BLM-1A was epithelioid-like, and BLAbenceous revertant fibroblasts. (Fig. 1A, F).

Fig. 1. Morphology of Fx/EJ or Fx+E/J transfectants as shown in culture and photographed by phase-contrast microscopy.

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lines and the E1A transfectants. E1A protein levels in the transfected cells were checked by immunoprecipitation (not shown) and corresponded with mRNA expression (Fig. 2A).

Expression of melanoma associated progression marker genes in the transfectants. For further characterization, RNA was analyzed from the non-metastatic cell line 1F6, the highly metastatic cell lines MV3 and BLM, and the transfected cell lines, BLM.mock, BLM.neo, BLM.E1-1, BLM.E1-2 and BLM.E1A (after transfection of E1A). G418 resistant cell clones were grown in sufficient amounts to allow RNA isolation without intermediary freezing of cells). As expected, E1A mRNA expression was only detectable in the three positive transfectants, BLM.E1-1 displaying the highest expression levels and BLM.E1A the lowest, hardly visible (Fig. 2A). In addition, we examined the expression of a panel of positive progression marker genes, including calcyclin (4), thymosin β10 (5), TGase2 (28), PAI-1 (30), PAI-2 (31),
FIG. 2. Northern blot analysis of human melanoma cell lines, and E1A or E1A-E1B transfectants. Ten μg of total RNA was loaded in each lane. Lane 1, 1F6; lane 2, MV3; lane 3, BLM; lane 4, BLM.mock; lane 5, BLM.neo; lane 6, BLM.E1-1; lane 7; BLM.E1-2; lane 8, BLM.E1A. (A) Expression of E1A, PAI-1, PAI-2, uPA, tPA, TGase2. (B) Expression of memA, memB, thymosin β10, calcyclin and c-myc. (C) Expression of p53, nma, nmb and ME491. (D) Expression of hsp70, β actin and vimentin. Lambda HindIII was used as a molecular marker; a 18S ribosomal probe was used for control hybridization.
TABLE I

<table>
<thead>
<tr>
<th>Cell line</th>
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<tr>
<td>530</td>
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</tr>
<tr>
<td>1F6</td>
<td>&lt;0.004</td>
</tr>
<tr>
<td>MV3</td>
<td>13</td>
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<tr>
<td>BLM</td>
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<tr>
<td>BLM.neo</td>
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<tr>
<td>BLM.E1-1</td>
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</tr>
<tr>
<td>BLM.E1-2</td>
<td>&lt;0.006</td>
</tr>
<tr>
<td>BLM.E1Arev</td>
<td>8.4</td>
</tr>
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</table>

Note. Cell lines were cultured for 48 hours, after which the cell density was determined and cell free supernatant assayed for IL-6 protein. IL-6 values are given as pg active protein per ml per 10^6 cells.

uPA (30), tPA (31), memA and memB (two novel genes, associated with highly metastatic melanoma cell lines; J. J. M. van Groningen, unpublished results), vimentin and c-myc (29) (note that c-myc showed no differential mRNA expression in our panel of human melanoma cell lines). The expression profile of BLM.E1-1, BLM.E1-2 and BLM.E1A showed a specific switch off for most of these genes, while thymosin β10 and vimentin were downregulated to the levels of the non-metastatic cell line 1F6. The expression of some other genes was hardly or not changed, notably β-actin, p53, nma and nmb (two novel genes, not expressed in our highly metastatic cell lines (7,9)), and the reverse progression marker ME491 (33). In accordance with earlier reports, the activity of hsp70 was greatly increased in E1A expressing cells (35). In the BLM.E1Arev cell line the expression of positive progression marker genes is restored to the levels of parental BLM (not shown). Gene activity in the non-metastatic cell line 530 corresponds with progression marker expression of cell line 1F6 (not shown).

**IL-6 production.** The secretion of IL-6 into the growth medium by the reference cell lines and the E1A transfectants was assayed by hybridoma growth stimulation (Table 1). The non-metastatic cell lines 1F6 and 530 did not produce IL-6, whereas the highly metastatic cell lines MV3 and BLM secreted significant amounts of IL-6 into the growth medium. IL-6 production by BLM.E1-1 and BLM.E1-2 was down-regulated to the level of the non-metastatic cell lines 1F6 and 530 when compared to parental BLM, BLM.neo and BLM.mock. IL-6 production by the BLM.E1Arev was at the level of the parental BLM cell line.

**Oncogenicity in nude mice.** The parental cell line BLM, two neo transfectants BLM.neo7 and BLM.neo12, and the cell lines BLM.E1Arev, and the E1A expressing BLM.E1-1 and BLM.E1-2 were used for evaluation of their xenograft development in nude mice. The parental cell line BLM, the two neo transfectants and BLM.E1Arev formed large tumors within 6 weeks. By contrast, the sizes were minimal of the xenografts formed by the BLM.E1-1 and BLM.E1-2 cell lines in this period (Fig. 3). These observations indicate that tumor growth of the E1A+E1B transfectants was suppressed.

**DISCUSSION**

The presented set of experiments was designed to study molecular mechanisms controlling the development of human cutaneous melanoma. After the introduction of Ad5 E1A gene into
the highly metastatic human melanoma cell line BLM by stable transfection, we have observed
the apparently collective and coordinate suppression of a panel of genes whose expression is
normally positively related with the malignant BLM phenotype. While some of these positive
progression markers have a proven functional relation with metastasis, the functions of some
others, including two novel genes, are unknown in regard to neoplastic progression. The
apparently coordinate type of intervention is remarkable and novel, and provides evidence for
the existence of a common pathway of neoplastic progression in melanoma cells.

Significant differences existed in the levels of ElA expression in the three transfectants,
BLM.E1-1 displaying the highest levels of both mRNA and protein. Despite these variations
all three ElA expressing transfectants were equally capable of suppressing positive progression
marker gene expression and changing cell morphology. As additional confirmation for the
presence of ElA activity we observed the well described stimulation of hsp70 expression (35)
in all three transfectants, BLM.E1-2 and BLM.E1A. The complete suppression of
several genes (e.g. u-PA, PAI-1) in all transfected cell clones is a strong argument in favor
of homogeneity of the cultures analyzed. The presence of the ElA gene had no effect on p53
expression, but it is known that ElA stabilizes the p53 tumor suppressor protein and
promotes apoptosis (36). Continuous culturing may, therefore, select for cells that have lost
ElA expression. Accompanying the loss of ElA expression, BLM.E1Arev had reverted to the
parental phenotype, further confirming that ElA activity is responsible for the observed phenoe-
typic changes. The Ad5 E1B gene products inhibit ElA associated apoptosis (37). Therefore,
the negative selection pressure against ElA expressing cells should be relieved in ElA+E1B
transfectants and, indeed, ElA expression is apparently stable in BLM.E1-1 and BLM.E1-2
cell lines.

In addition to the differences in the gene expression profiles, the ElA transfectants display
changes in cell morphology and cytoskeletal organization. This phenomenon is not normally
related with melanoma progression (compare ref. 38). Apparently, ElA proteins do not only
cause suppression of late progression marker genes but have broader effects. This is not
surprising in view of the number and nature of the cellular factors that are known to associate

FIG. 3. Growth curves of the tumors from parental BLM, BLM.neo 7, BLM.neo 12, BLM.E1Arev, BLM.E1-1 and
BLM.E1-2 cell lines in nude mice. Approximately $3 \times 10^6$ cells were inoculated s.c. into nude mice. Tumor volumes
were measured weekly and mice were allowed to sit for three months unless the tumor size interfered with the animal's
health. Values are given as a mean of all animals (5 animals per cell line) tested.
with E1A (11,39). E1A induced phenotypic alterations were more often associated with a cytoskeletal reorganization, the resultant patterns being cell-type-dependent (38,40).

Our data provide new evidence in support of a tumor suppressor function for E1A, as suggested recently by several other groups (12,40-43). Reduced oncogenicity would be a likely and logical consequence of the observed suppression of positive progression marker genes. Subcutaneous inoculation of the transfected melanoma cells into nude mice indeed showed that the presence of E1A significantly reduced tumor growth. The properties of the reverted cell line BLM.E1Ar corresponded also in this test with those of parental BLM.

How does Ad5 E1A suppress gene transcription? Despite the large number of studies devoted to this subject no unifying concept has emerged yet. The ample data are listed in recent reviews (11,39). In general, it is concluded that the mechanism does not seem to be mediated by one specific factor or interaction. Furthermore, it is pointed out that the qualitative effect (suppression or promotion) is dependent on the cell type used, indicating that the identity of transcription factors present plays an essential role. Various domains of the E1A proteins interact with several factors (RB, p107, p130, p300). It is known that E1A inhibits cAMP-dependent activation of the IL-6 promoter due to interactions with and inhibition of CBP (44). The mechanisms of the effects described in this report have to be elucidated in further studies.

In summary, our results add new evidence to the earlier observed suppression/reversion of neoplastic progression by E1A (15,17,40,42,43,45). More importantly, while the apparent inhibition of the oncogenic potential of several human tumor cell lines was ascribed to the mere suppression of individual genes essential for advanced progression, our data show that E1A interference is more comprehensive and concerns the specific suppression of all tested genes whose expression is positively correlated with neoplastic progression in our panel of human melanoma cell lines of increasing metastatic potential. We therefore conclude that E1A fulfills the role of a "master gene", i.e. a gene responsible for the transition of one phenotype to another. The coordinate intervention realized by a viral gene suggests that neoplastic progression proceeds along a common pathway(s), which may be equally well controlled by (an) as yet unidentified cellular factor(s).

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