E2F-1 Gene Transfer Enhances Invasiveness of Human Head and Neck Carcinoma Cell Lines

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Abstract

The transcription factor E2F-1, a downstream regulator of the p16-cyclinD-Rb pathway, is required for cell cycle progression. Evidence shows that overexpression of E2F-1 can either promote or inhibit development of tumors, depending on tissue or experimental conditions. To study whether the E2F-1 gene plays a role in tumor progression, the expression of E2F-1 protein was evaluated in 10 human head and neck squamous cell carcinoma cell lines using Western blot analysis. In addition, the invasive ability of these cell lines was determined by evaluating the penetration of cell lines into the tracheal wall in an in vivo invasion assay using dermepithelialized trachea transplanted into the s.c. tissue of SCid mice. This study showed that the aggressive cell lines had higher expression of E2F-1 than the less invasive cell lines. To evaluate the hypothesis that E2F-1 enhances invasiveness, we selected two cell lines, SCC9 and SCC12, for a gene transfer experiment. These cell lines exhibited low invasive ability with low expression of E2F-1. Two stable clones with overexpression of transfected E2F-1 gene and two clones with their respective vector-alone control were selected from each cell line for in vivo invasion evaluation. The clones containing the transfected E2F-1 gene had significantly higher invasive ability than their respective vector-alone clones. Flow cytometry showed that parental, transfected E2F-1, and vector-alone cells had a similar proliferation pattern under normal culture conditions. Nevertheless, transfected E2F-1 cells exhibited a higher portion of cells in S phase than the control cells after serum-starvation and refeeding. The results indicated that overexpression of E2F-1 plays a positive role in cell cycle reentry from quiescence and is associated with increased in vivo invasiveness.

Introduction

Mammalian cell proliferation is a process controlled by a very complex cell cycle machinery. Multiple components such as cyclins, Cdk inhibitors, Rb, E2Fs, etc., are implicated in the transitions from one cell cycle phase to another. For example, D-type cyclins bind and activate Cdk4/Cdk6, which phosphorylate the Rb gene product pRb, whereas Cdk inhibitors such as p21 and p16 can inhibit the function of Cdk5. As a result of phosphorylation of pRb, E2F is released from the pRb-E2F complex. The freed E2F is an active transcription factor that promotes the transcription of the genes required for DNA synthesis and drives cells from the G1 phase into the S phase (1–3).

Any deregulation of these events may cause uncontrolled cell cycle and lead tumor development. Evidence shows that the alterations of the cyclin-Cdk-Rb pathway are involved in the development of most human malignancies. For example, amplification and overexpression of cyclin D1, have been described in several tumor types, including 30 to 50% of HNSCC (4–6). At least one alteration of p16, cyclin D1, or Rb was detected in up to 91% of primary HNSCC (7).

The transcription factor E2F-1 is the best-known ultimate transcription factor activated in the cyclin-Cdk-Rb pathway. In vitro experiments showed that E2F-1-binding activity and mRNA level fluctuate through the cell cycle, peaking at the G1-S phase boundary. Overexpression of E2F-1 can drive quiescent cells into S phase and induce G1-arrested cells into S phase, even in the absence of Cdk activity (8, 9). Abnormalities in E2F-1 gene expression and/or gene amplification have been described in several tumor types including gastrointestinal and lymphatic tumors (10, 11). In addition, other studies demonstrated that normal keratinocyte growth arrest in vitro is associated with a destabilization of E2F-1 mRNA. Conversely, SCC cell lines had a very stable E2F-1 half-life and did not exhibit E2F-1 down-regulation in response to growth inhibitors (12, 13). In contrast, E2F-1 gene transfection induced apoptosis in some cell lines (14–16) and E2F-1 knockout mice showed a predisposition to develop spontaneous tumors (17). Clinical follow-up studies showed that low E2F-1 expression was associated with adverse outcome in bladder cancer, but overexpression of E2F-1 was associated with high-grade tumors and poor outcome in breast cancer (18, 19). A recent study indicates that there is a trend toward increased E2F-1 expression levels in metastatic progression of colorectal carcinoma (20). Thus, it is feasible that E2F-1 participates in the process of tumor development and progression in several tumor types.

To study whether the E2F-1 gene plays a role in tumor progression, the expression of E2F-1 protein and invasive ability was evaluated in SCC cell lines of different degrees of invasiveness. Furthermore, to confirm the hypothesis that E2F-1 enhances tumor progression, we selected two cell lines of low invasive potential for an E2F-1 gene transfer experiment. The results indicate that overexpression of E2F-1 is associated with increased tumor cell invasiveness.

Materials and Methods

Squamous Carcinoma Cell Lines of the Head and Neck. The 10 human HNSCC cell lines were used in this study. They were SCC12, SCC13, SCC9, SCC15, SCC25, SCC40, SCC71, A253, Detroit 562, and FADU. All SCC series of cell lines were provided by Dr. J. Rheinwald, the rest of the cell lines were obtained from the American Type Culture Collection. The cells were cultured in S-MEM supplemented with 10% fetal bovine serum.

Western Blot Analysis. Subconfluent cell cultures were washed three times in ice-cold PBS and then lysed with lysis buffer as described previously [Ref. 21; 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% NP 40, 50 mM NaF, 1 mM Na3VO4, 1 mM DTT, 1 mM PMSF, and 25 μg/ml of trypsin inhibitor]. After incubation for 30 min, cell lysates were clarified by centrifugation at 13,000 rpm for 5 min and protein concentration of the supernatants was determined with Bio-Rad DC protein assay (Bio-Rad, Hercules, CA). Eighty μg of total protein was loaded on each lane. The samples were subjected to 7.5–12% SDS-PAGE and then transferred to nitrocellulose membrane and

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3 The abbreviations used are: Cdk, cyclin-dependent kinase; Rb, retinoblastoma; SCC, squamous cell carcinoma; HNSCC, head and neck squamous cell carcinoma; S-MEM, S-minimal essential medium ; RSV, Rous sarcoma virus.

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immunoblotted with the following antibodies: mouse monoclonal antibody E2F-1 (SC-251); and Actin (SC-8432; Santa Cruz Biotechnology). The concentration of each antibody was used as suggested by the suppliers. Immunodetection was performed with the enhanced chemiluminescence kit for Western blotting (Amersham, Arlington Heights, IL).

**E2F-1 Gene Transfection.** RSV E2F-1 was constructed by inserting the human E2F-1 cDNA into the HindIII and XhoI sites of pReRSV (V780-20; Invitrogen, Carlsbad, CA; Ref. 24) with RSV long terminal repeat as a E2F-1 gene promoter and a neo gene as a selection marker. The RSVE2F1 or RSV were transfected into SCC9 and SCC12 cell lines with lipofectin (10964-013, Life Technologies, Inc.) as suggested by the manufacturer. Forty-eight h after transfection, 800 μg/ml of G418 was added to normal growth medium to select for the clones expressing the transfected neo gene. Then the stable clones were picked, expanded, and maintained using 200 μg/ml of G418 in the medium for further analysis.

**Northern Blot Analysis.** Total cellular RNAs from control and transfected HNSCC cell lines were isolated by the RNAzol method (Biotect) according to the manufacturer’s instructions. Twelve μg of total RNAs were size-fractionated on a 1% agarose gel containing 0.7 M formaldehyde and transferred onto HyBond N+ membranes (Amersham) with 10× SSC, rinsed briefly in 2× SSC, and vacuum-dried at 80°C for 2 h. The blots were hybridized with random-primed 32P-labeled cDNAs for E2F-1, neo, or β-actin (Oncogene) in ExpressHyb hybridization solution (Clontech) at 68°C, respectively.

**Flow Cytometry Analysis.** Exponentially growing cells were starved in S-MEM without serum for 72 h, then refed with S-MEM with 10% fetal bovine serum. The cells were harvested at different time points, washed with cold PBS, and fixed in 70% ethanol. The cells were stained with 50 μg/ml propidium iodide with 100 μg/ml RNase in PBS. DNA content and the percentage of cells in various phases of the cell cycle were determined by measuring fluorescent intensity with a Coulter Elite Flow Cytometer.

**In Vivo Invasion Assay.** A xenotransplantation system that permits the evaluation of tumorigenicity and in vivo invasiveness of human cell lines was used. This bioassay is based on the growth of tumor cells in deepithelialized rat tracheas transplanted s.c. into Scid mice and has been described previously (23). In brief, tracheas from Fisher 344 rats were deepithelialized by repeated freezing and thawing. After sealing one end, 5 × 105 cells in 100 μl of tissue culture medium were seeded into the lumen. Following s.c. transplantation, eight tracheal transplants from each cell line were removed at 8–12 weeks. Remodeling of the tracheal wall. The invasion of the tracheal wall was classified according to the level of penetration of the neoplastic cells into Levels 0–3. Level 0 included cells with no invasion of the tracheal wall, in which the cells were confined to the lumen. Level 1 cells showed a low invasive ability characterized by cell penetration into the superficial lamina propria. In level 2, cells invaded into the deeper lamina propria, including the pars membranacea. Finally, Level 3 is characterized by cells invading deeply into the tracheal wall and reaching the tracheal adventitia.

**Immunohistochemistry.** Formalin-fixed and paraffin-embedded sections of tracheal transplants containing transfected HNSCC tumor cell lines were used for the immunohistochemical detection of E2F-1 and Ki-67 (Mib-1). A mouse monoclonal antibody to E2F-1 (Santa Cruz, CA), a mouse monoclonal Mib-1 (Immunotech, Westbrook, ME), and an avidin-biotin-peroxidase kit (Immunotech, Westbrook, ME), and an avidin-biotin-peroxidase kit used for the immunohistochemical detection of E2F-1 and Ki-67 (Mib-1). A few number of cells could be seen lining the tracheal lumina.

**Results**

**Invasive Ability of SCC Lines.** An in vivo assay of tumor cell invasiveness based on the evaluation of tumor cell penetration into the tracheal wall of tracheal xenotransplants was used to determine the invasive ability of HNSCC cell lines. Five cell lines that had extensively invaded the adventitia and surrounding s.c. tissue at 8 weeks after transplantation were classified as highly invasive (level 3); whereas the four cell lines that had penetrated only the lamina propria of the tracheal graft were classified as of low invasiveness (levels 1 and 2). Among the latter, SCC13 and SCC25 were only marginally invasive and were almost always seen lining the lumen and forming a stratified epithelium that did not penetrate into the lamina propria (level 0). SCC15 and SCC9 showed similar behavior, although a large number of cells could be seen in the subluminal tissue. SCC12 cells grew with difficulty in this xenotransplantation system, and a few cells could be seen lining the tracheal lumina.

The highly invasive cell lines SCC40, SCC71, A253, Detroit 562, and FADU invaded the entire thickness of the tracheal wall including the pars membranacea and the adventitia at 8 weeks after transplantation. All cell lines with high invasive ability produced moderately-to-poorly differentiated squamous carcinomas; whereas all cell lines of low invasiveness resulted in well- to moderately differentiated tumors.

**Expression of E2F-1 Protein in HNSCC Cell Lines.** Total protein lysates were made from each of the cell lines. Protein expression in HNSCC was determined by Western analysis. The HNSCC cell lines with the higher expression of E2F1 were SCC13, SCC40, SCC71, Detroit 562, and FADU; all cell lines except SCC13 exhibited high invasive ability. Most cell lines characterized by low invasive potential, such as SCC9, SCC12, SCC15, and SCC25, showed lower expression of E2F1 protein. Nevertheless, one highly invasive cell line, A253, exhibited low levels of protein expression (Fig. 1A).

**Transfer of the E2F-1 Gene into HNSCC Cell Lines.** Because overexpression of E2F-1 seemed to be associated with a higher invasive ability in most HNSCC cell lines, an experiment of E2F-1 gene transfer into cells of low invasive ability was performed. SCC9 and SCC12, two cell lines with these characteristics and expressing relatively low levels of E2F1 protein, were selected for this purpose. The human E2F-1 cDNA expression plasmid RSV/E2F1 and the control vector Re/RSV were transfected into SCC9 and SCC12 cells.
The colonies growing after G418 selection were picked and expanded. Western and Northern blot analysis of the picked clones revealed different levels of E2F-1 expression. Two clones containing transfected RSV/E2F1 with the highest expression were selected from each cell line for further analysis. These two clones from each cell line were designated as SCC9/E2F1a, SCC9/E2F1b and SCC12/E2F1a, SCC12/E2F1b, respectively. The respective controls containing vector alone were designated as SCC9/RSVa, SCC9/RSVb and SCC12/RSVa, SCC12/RSVb, respectively. Both clones from each cell line expressed E2F1 at higher levels than their respective controls, as shown by either Northern analyses or Western blot (Fig. 1B, 1C). An immunohistochemical study revealed that the clones transfected with E2F-1 had a higher number of stained cells and more intense nuclear staining than their respective controls. The latter showed no or marginal staining for E2F-1 (data not shown).

**Overexpression of E2F1 Enhances Invasiveness of HNSCC Cell Lines.** An in vivo invasion assay was performed using tracheal xenotransplants containing cells that were transfected either with the E2F1 gene or with vector alone. Levels of invasiveness were evaluated 12 weeks after xenotransplantation. SCC9/E2F1 cells were more invasive than SCC9/RSV cells with 11 of 16 and 2 of 15 tracheal transplants reaching the highest invasion levels (levels 2 and 3), respectively (Fig. 2). There was a statistically significant difference between E2F-1 transfectants and vector-alone transfected cells \((P < 0.001)\). A similar difference was seen between the SCC12/E2F1 cells and the SCC12/RSV cells \((P < 0.05)\) with 8 of 15 and 1 of 15 tracheal transplants reaching the highest invasion levels, respectively. The immunohistochemical detection of E2F-1 in tracheal xenotransplants showed that the invasive E2F-1 transfectants had higher expression of the E2F-1 protein than the vector-alone transfected cells (Fig. 3, A and B).

**Effect of E2F1 on HNSCC Cell Proliferation.** Proliferation changes were evaluated in vivo using the immunohistochemical detection of the Ki67 antigen (Mib-1) in tracheal xenografts. The Mib-1 nuclear staining indices of vector-alone and E2F-1-transfected cells in tracheal transplants were similar, i.e., 46.7% for SCC9/RSVa cells, 48.3% for SCC9/E2F1a cells, 45.6% for SCC12/RSVa cells, and 44.7% for SCC12/E2F1a cells (Fig. 3, C and D).

The in vitro growth patterns and doubling times of these cell lines were not significantly different. Nevertheless, cell proliferation was...
also examined by flow cytometry using cultured cells. Under normal feeding conditions, the percentage of transfected cells in S phase and their respective controls were not statistically different. Nevertheless, after serum starvation and refeeding, some differences were observed. The E2F-1 transfected clones had a higher percentage of cells in S phase than their respective controls 36 h after refeeding. For example, SCC9/RSVa and SCC9/RSVb cells in S phase showed a 2-fold or higher increase compared with the same cells fed under normal serum conditions, whereas the E2F-1-transfected cells, SCC9/E2F-1a and SCC9/E2F-1b, exhibited a 3- to 4-fold increase in the percentage of S-phase cells than the same cells fed normally. Similar differences were observed between the E2F-1-transfected SCC12 cells and their respective control transfected cells.

Discussion

E2F-1 is the best-known transcription factor regulated by the cyclin-Cdk-Rb pathway. E2F-1 was the first cloned and is the better-characterized member of this gene family (8, 24). Several lines of evidence indicate that E2F-1 is involved in neoplastic development. For example, overexpression of E2F-1 was shown to induce neoplastic transformation in cultured fibroblasts (22). In addition, amplification of the E2F-1 gene was found in an erythroleukemia cell line (25), in 4% of gastric carcinomas, and in 25% of colorectal carcinomas (10). Furthermore, increased expression of E2F-1 mRNA was observed in 40% of gastric carcinomas and in 60% of colorectal adenocarcinomas (10). A recent study showed that loss of E2F-1 reduced the frequency of pituitary and thyroid tumors in Rb1 (-/-) mice (26). Recently, immunohistochemical studies have demonstrated that E2F-1 is overexpressed in some non-Hodgkin’s lymphomas (11) and in a large percentage of breast invasive ductal carcinomas (19). Increased E2F-1 expression was found in metastases of colorectal carcinomas (20). In contrast, E2F-1 gene transfection induced apoptosis in some cell lines (14-16) and E2F-1 knockout mice developed a variety of tumors (17). A recent study of E2F-1 in the bladder cancer showed that patients with lower E2F-1 protein expression had adverse outcome (18). These findings suggest that E2F-1 could function either as an oncogene or a tumor suppressor gene depending on tissue type and experimental conditions.

To our knowledge, there are no examples of E2F-1 abnormalities in any HNSCC tumors or cell lines. In the present report we have shown that E2F-1 is overexpressed in HNSCC cell lines that show an enhanced malignant phenotype as determined in an in vitro invasion assay. The fact that this is also accompanied by a low level of expression of this cyclin in the HNSCC lines of low invasive potential strongly suggests an association with the aggressive phenotype. In addition, we demonstrated that E2F-1 transfer into two HNSCC of low invasive potential in the same in vitro invasion assay confers to these cells an enhanced invasive ability. Interestingly the transfer of the E2F-1 gene did not change the in vitro proliferative characteristics of the two HNSCC cell lines as demonstrated by the absence of labeling index differences in tracheal transplants immunostained for Ki-67 (Mib-1). Conversely, in vitro the transfected cells were able to enter the cell cycle in larger numbers after the serum deprivation induction of quiescence. Under normal feeding conditions the in vitro proliferative capacity and growth behavior was unchanged by E2F-1 transfection.

Although, gelatinolytic activity was not markedly increased by E2F-1 transfection (data not shown) it is quite possible that certain invasion-associated genes such as metalloproteinases and cell adhesion proteins and their regulators might be directly responsive to the E2F-1 transcription factor. An indirect association through a more complex cascade is also possible. For example, recent findings originally described in yeast are pointing to a probable cross-talk between the cell cycle machinery and certain cell functions such as cell-cell contacts and cell-matrix interactions. These gene products as well as the recently described p21 Cas-related gene HEF-1 (27), could shuttle between nucleus and cytoplasm and interfere in focal adhesion and other cytoplasmic functions such as secretion of metalloproteinases. In addition, in a recent report Li et al. (28) describe the inhibition of cell invasiveness after replacement of the Rb gene in Rb-defective tumor cells.

The relationship between E2F-1 expression and cell invasiveness has been determined functionally using gene transfer experiments. Whether E2F-1 and associated gene products can regulate directly or indirectly the expression of metalloproteinases or other genes involved in tumor progression, as suggested by Li et al. (28) is still a matter of speculation and will require additional work.

References


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