Ionizing Radiation and TNF-α and Stimulated Expression of α1-Antichymotrypsin Gene in Human Squamous Carcinoma Cells

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α-1 antichymotrypsin (ACT), a serine protease inhibitor, has been detected in several epithelial tumor cell types, but its role in response to therapy is not clear. We report here that exposure of primary head and neck squamous cell carcinoma (HNSCC)-derived cells (PCI-04A) to ionizing radiation (IR) or tumor necrosis factor-α (TNF-α) resulted in an increased level of ACT mRNA, although the induction patterns were different. IR treatment caused a transient stimulation of ACT mRNA, peaking at 3 h post-irradiation, whereas TNF-α-inducible ACT gene xpression lasted for up to 24 h. The ACT mRNA was expressed in several epithelial and non-epithelial tumor cell types, and in different normal human tissues. In addition, when the ACT gene expression in PCI-04A cells was compared with the matched (from the same patient) metastatic HNSCC-derived cells (PCI-04B), increased steady-state level of the ACT mRNA was observed in PCI-04B cells. Taken together, these findings suggest that ACT may serve as an important marker for prognosis and therapy selection in HNSCC.

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Human α1-antichymotrypsin (ACT) is a member of the serine protease inhibitor (Serpin) superfamily which regulates the activity of several serine proteases including chymotrypsin and cathepsin G (1, 2). It is one of the acute-phase proteins that rise rapidly in response to injuries such as infection, trauma, and malignancies, apparently providing protection and preventing damage (3, 4). The mechanism of increase has been related to levels of cytokines (IL-1, IL-6, TNF-α) and TGF-α1 (3, 5). Insulin-like growth factors and estradiol have been shown to increase the expression of ACT in MCF7 human breast cancer cells (6). Our recent findings suggest that stimulation of the ACT gene expression in antisense *c-raf-1* cDNA transfected squamous carcinoma cells may be a protective response (7). ACT has also been shown to inhibit cell proliferation by binding to enzymes associated with DNA replication such as DNA polymerase (8). Serpins are also known to inhibit free radical production, and apoptosis by ionizing radiation (IR) (9). To date, however, a role of ACT in tumor cell response to IR has not been investigated. In this study, we determined the effect of IR or TNF-α on the ACT gene expression in human primary head and neck squamous carcinoma-derived cells (HNSCC) (PCI-04A) (10), and we assessed the constitutive levels of the ACT mRNA in different human tumor cell types and normal tissues. Proteases have been proposed to play a role in tumor metastasis (11), and it is likely that serpins may function in the regulation of metastasis. Theoretically, comparison between the primary and matched (from the same patient) metastatic tumor cells should minimize individual genetic variations. As a step towards an understanding of the part played by ACT in metastasis, here we also compared the expression of ACT gene in PCI-04A cells with the matched metastatic tumor-derived cells (PCI-04B) (10).

MATERIAL AND METHODS

Cell culture

The tumor-derived cell lines were grown in minimum essential medium supplemented with 15% heat-inactivated fetal bovine serum (FBS), 10 mM HEPES (pH 7.2), 1 mM non-essential amino acids, 2 mM L-glutamine, 25 μg/ml gentamicin sulfate, all obtained from GIBCO/BRL, and 0.5 μg/ml hydrocortisone (Sigma). Cells were cultured at 37°C and under 5% CO_2.

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Radiation and TNF-α treatments

Logarithmically growing cells were switched to serum-free medium and grown overnight prior to irradiation (15 Gy, 114 cGy/min), as we described earlier (12). Following irradiation, the cells were returned to the incubator and RNA was isolated at various times post-irradiation. In TNF-α experiments, cells were incubated for 1 h in medium containing 1% heat-inactivated FBS, and then TNF-α (R and D Systems) was added to a final concentration of 100 ng/ml. Incubations continued at various times prior to RNA isolation.

Northern blot analysis

Total RNA was isolated by the guanidium isothiocyanate method (13), fractionated on a 1.0% formaldehyde agarose gel, and transferred onto nitrocellulose membrane. Human ACT partial cDNA (7), and GAPDH cDNA probes were labeled with [α-^{32}P]dCTP using a random primer DNA labeling kit (Pharmacia Biotech). Blots were sequentially hybridized first to a radiolabeled partial human cDNA insert and then to a GAPDH cDNA probe at 42°C in buffer containing 50% formamide, 5X SSC, 1X Denhardt's solution, 20 mM sodium phosphate (pH 6.8), and 200 μg/ml sheared and denatured salmon sperm DNA. Blots were washed three times in 2X SSC and 0.1% SDS, five times in 0.2X SSC and 0.1% SDS, and twice in 2X SSC at 55°C. Dried blots were exposed to x-ray films. Autoradiographs were computer-scanned using the Image-Quant software (Molecular Dynamics). The statistical significance of the differences in gene expression between the primary and matched metastatic tumor cell lines was determined using the paired *t* test. ACT gene expression was also examined on poly $(A)^+$ mRNA blots of human multiple normal tissues and other cancer cell lines (Clontech). These blots were sequentially hybridized with the cDNA insert followed by the β-actin cDNA probe. Prehybridization and hybridization were carried out in ExpressHyb solution (Clontech) at 68°C, and the blots were washed in several changes of 0.1% SDS, 2X SSC for 40 min and 0.05% SDS and 0.2X SSC for 20 min. Damp blots were covered with plastic wrap and exposed to x-ray film at -70°C.

RESULTS AND DISCUSSION

Effects of ionizing radiation and TNF-α on the ACT gene expression in PCI-04A cells

We examined the effects of two known stress inducers, IR and TNF-α, on the ACT gene expression in PCI-04A cells. IR or TNF-α treatment led to stimulation of the ACT gene expression. However, the kinetics of responsiveness indicates that the mechanisms of IR and TNF-alpha-induced signaling are distinct. IR stimulated the steady-state level of ACT mRNA, and the maximal level was observed at 3 h after IR (Fig. 1), whereas TNF-α treatment caused a relatively prolonged stimulation of ACT mRNA observed up to 24 hr (Fig. 2). Quantitative analysis indicated ~2-fold increase in the level of ACT mRNA at 3 h in irradiated cells, whereas a ~2–7-fold increase in the ACT gene expression was observed at 4 h and 24 h following TNF-α treatment.

The precise mechanism of induction of ACT gene expression is unclear. It is well documented that TNF-α causes activation of NF-κB (14). We have also demonstrated that exposure of PCI-04A cells to IR results in the activation of MAPK signal transduction pathway, and increased DNA binding activities of several transcription factors including NF-κB (12, 15, 16). Whether induction of the ACT gene expression requires NF-κB activity in PCI-04A cells remains to be seen. PCI-04A cells are moderately sensitive to TNF-α and IR (17, 18) (UK, unpublished data). In addition, protease inhibitors are known to suppress apoptosis induced by cytotoxic agents (9, 19). Therefore, one possibility is that the increase in the ACT gene expression may be a damage-inducible protective response in HNSCC. It is noteworthy that TNF-α and radiation induce a cascade of both cytotoxic and cytoprotective genes (20, 21). The biological response would then

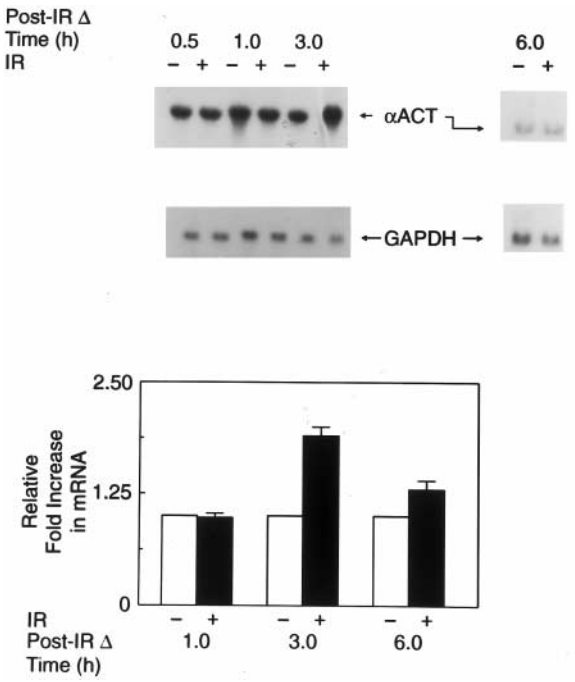

Fig. 1. Effect of IR on the ACT gene expression in PCI-04A cells. Top panels: Cells were exposed to IR (15 Gy, 114 cGy/min), followed by incubation for indicated times and the ACT gene expression was analyzed by Northern blot hybridization. Blots were subsequently reprobed with GAPDH cDNA probe. Bottom panel: Computerized densitometric scanning analysis from two to three independent experiments. The relative fold increase in ACT mRNA level was determined by first normalizing the ACT signal against the corresponding GAPDH signal, and then comparing the level of ACT gene expression in irradiated (+) samples relative to unirradiated (−) samples at each time point.

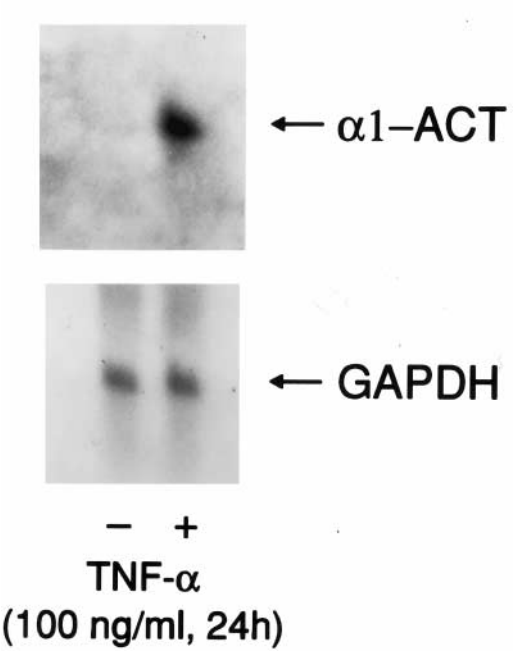

Fig. 2. Effect of TNF-α on the ACT gene expression in PCI-04A cells. Cells were treated with TNF-α as indicated, and the ACT gene expression was examined by Northern blot hybridization. Blots were reprobed with GAPDH cDNA probe. Data shown is a representative of three independently performed experiments. Autoradiographs were computer-scanned, and the relative fold increase in ACT gene expression was determined as explained in Fig. 1.

be determined by a balance of the toxic and protective signals, perhaps in a cell-type-specific manner.

Expression of the ACT gene in normal human tissues and other tumor cell lines

Varying levels of the ACT gene expression were observed in different normal human tissues, with a relatively very high level in liver (~ 12-fold), and very low levels in heart (~ 0.77-fold), and lung (~ 1.08-fold) (Fig. 3A). In addition, other tumor cell lines tested showed significant expression of the ACT gene (Fig. 3B). High expression of the ACT gene was also found in a human melanoma cell line (data not shown). Previously, liver has been reported as the major source of ACT synthesis (22). Present studies extend these findings, and demonstrate that several other normal tissues, and hematopoietic and non-hematopoietic tumor cells express significant levels of the ACT mRNA. These data are consistent with a part played by serpins in the control of a wide range of physiological activities modulated by proteolytic enzymes including blood coagulation, implantation, inflammatory stimuli, and complement activation.

Expression of the ACT gene in matched primary and metastatic HNSCC-derived cell lines

We compared the expression of ACT gene in the primary

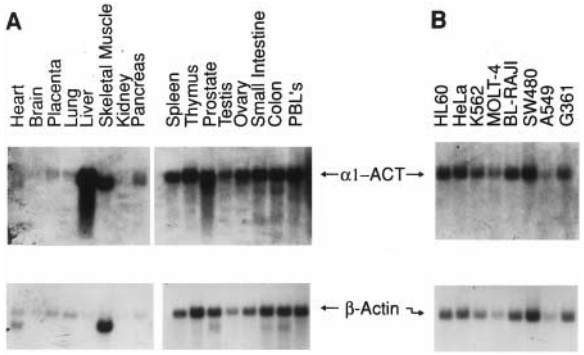

Fig. 3. Expression of ACT in normal human tissues (panel A) and human cancer cell lines (panel B). Northern blots were sequentially hybridized with a radio-labeled partial human ACT cDNA probe (7), followed by β-actin cDNA probe. HL60, promyelocytic leukemia; K-562, chronic myelogenous leukemia; Molt-4, lymphoblastic leukemia; BL-RAJI, Burkitt's lymphoma; SW40, colorectal adenocarcinoma; A549, lung carcinoma; G631, melanoma.

HNSCC-derived PCI-04A cells with the matched metastatic HNSCC-derived PCI-04B cells. Both cell lines were established from tumor biopsies without prior therapy (10). Northern blot analysis indicated that the ACT gene expression was higher (~ 37%) in PCI-04B cells compared with PCI-04A cells (Fig. 4). These data were found to be statistically significant ($p < 0.0128$, $n = 4$). Similar results were obtained when another set of matched primary and metastatic HNSCC lines (PCI-15A and PCI-15B) was analyzed. The ACT gene expression was ~ 30% higher in metastatic tumor-derived cell line, PCI-15B, compared with the matched primary tumor cell line, PCI-15A (data not shown).

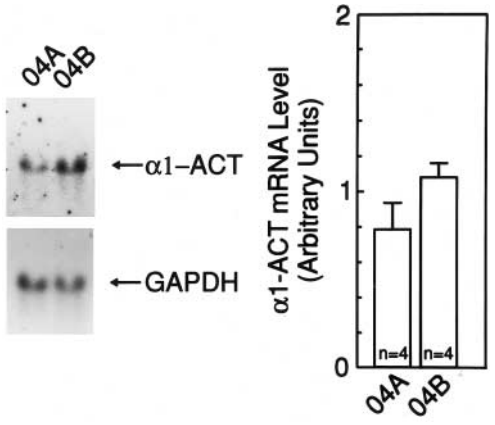

Fig. 4. Steady-state levels of the ACT gene expression in PCI-04A and PCI-04B cell lines. Left panel: Total RNA was isolated and mRNA expression was analyzed by the Northern blotting and hybridization. Right panel: Computerized densitometric scanning analysis of the ACT mRNA. Data were normalized against the GAPDH signal on the same blot.

In summary, modulation of ACT gene expression in response to stress and during metastasis provides new insights into the diverse mechanisms of ACT gene regulation, and suggests that ACT may serve as an important marker for prognosis and therapy selection in HNSCC. These findings establish a basis for future investigations of ACT expression in clinical tumor specimens, and the association of the active form of ACT with its physiological substrate in HNSCC.

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