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CYTOSKELETAL CHANGES DURING RADIATION-INDUCED NEOPLASTIC TRANSFORMATION OF HUMAN PROSTATE EPITHELIAL CELLS

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Abstract

We recently reported tumorigenic transformation of SV40-immortalized neonatal human prostate epithelial cells (267B1) by exposure to fractionated doses of X-rays. Altered morphology and anchorage independence were observed following two successive fractions of 2 Gy each (F3-SAC). Additional 2 Gy treatments to these non-tumorigenic cells to a total dose of 30 Gy resulted in radiation-transformed tumorigenic colonies (267B1-SXR). Malignant transformation of parental 267B1 cells was also achieved by consecutive 2 Gy exposures to a total dose of 30 Gy (267B1-XR). This study discusses the cytoskeletal changes in the F3-SAC, 267B1-XR and 267B1-SXR derivatives of these human prostate epithelial cells. Confocal and conventional fluorescence microscopy of filamentous actin showed numerous, well organized, evenly distributed stress fibers in the parental cells prior to irradiation, while the anchorage-independent cells and several tumorigenic derivatives exhibited poor stress fiber organization after radiation exposure. This disorganization of actin microfilaments in the radiation-transformed cells was also accompanied by changes in the expression of selective tropomyosin isoforms as judged by two-dimensional gel electrophoresis. These changes in actin organization and tropomyosin expression appear to be coincidental with morphological transformation and acquisition of tumorigenicity in the 267B1 cells following radiation exposure.

Key Words: X-rays, human prostate epithelial cells, tumorigenic progression, neoplastic transformation, anchorage independence, actin cytoskeleton, tropomyosin.

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Introduction

Epidemiological studies of populations exposed to ionizing radiation have shown that a wide range of tumor types are associated with such exposure (Shall, 1984). The direct oncogenic effects of low-LET radiations (X-rays and γ-rays) have been reported using various *in vitro* transformation systems (Borek, 1982; Elkind *et al.*, 1991; Kennedy *et al.*, 1980; Watanabe *et al.*, 1984). Prostate cancer is among the most commonly diagnosed malignancies in American males, and the involvement of ionizing radiation in its development is unclear. The genetic and environmental factors responsible for the progression of prostate cancer are also largely unknown (Isaacs, 1993; Isaacs *et al.*, 1995). For most epithelial systems, the development of fully malignant tumor cells from target stem cells involves stable intermediate stages. The development of each stage during malignant transformation is accompanied by a variety of morphological, cytological, and biochemical changes. It is of particular interest to establish the involvement of ionizing radiation in the multistep process of prostate carcinogenesis. The present study examines the cytoskeletal changes in human prostate epithelial cells as a result of exposure to X-rays.

The structural elements of cytoskeleton consist of three major components: microtubules, microfilaments, and intermediate filaments. Over the past decade, immunofluorescence studies on cultured cells have led to the conclusion that microfilaments are composed of several proteins in addition to actin, including myosin, tropomyosin (TM), α-actinin, and filamin (Matsumura *et al.*, 1983). Many cell functions, such as maintenance of morphology, aggregation, motility, membrane ruffling, smooth muscle contraction, and cytokinesis are regulated by the dynamic reorganization of actin microfilaments (Pollard and Cooper, 1986; Tanaka *et al.*, 1993). In view of the diversity of the functions of the cytoskeleton, it is not surprising that there is increasing evidence for the involvement of cytoskeleton and microfilaments in transformation (Brinkley, 1982; Carley *et al.*, 1981; Gowing *et al.*, 1984; Leonardi *et al.*, 1982; Lin *et al.*,

1984; Matsumura *et al.*, 1983; Miller *et al.*, 1992; Tanaka *et al.*, 1993). Early studies by Weber *et al.* (1974) have shown that parallel arrays or bundles of microfilaments, called stress fibers, are reduced or absent in transformed cells. Stress fibers are composed primarily of actin and contain α-actinin, myosin, and tropomyosin. It still remains to be seen whether changes in the protein composition or the structural organization of microfilaments occur concomitantly with oncogenic transformation of the prostate epithelial cells, and in particular, the order of sequence of events.

Neoplastic transformation of human cells is a complex, multistep process. The difficulties encountered with *in vitro* transformation of human cell systems and usefulness of virally-immortalized cell systems for transformation studies have been previously reviewed (Rhim, 1989; Rhim *et al.*, 1993). We recently reported the neoplastic conversion of SV40-immortalized, neonatal prostate epithelial cells (267B1) (Kaighn *et al.*, 1989) with either v-Ki-*ras* (Parda *et al.*, 1993) or with fractionated doses of ionizing radiation (Kuettel *et al.*, 1996). Parental cells that received different total amounts of X-rays represent intermediate steps during progression to malignancy. These different stages were determined by criteria of immortality, anchorage (in)dependence, growth in semisolid medium, and tumorigenicity. In the present study, we determine whether cellular transitions developed during the progression towards a neoplastic phenotype in the 267B1 cells, initiated by either v-Ki-*ras* or ionizing radiation, result in similar changes in the actin cytoskeleton. Using confocal, as well as immunofluorescence microscopy and two-dimensional gel electrophoresis (2D-PAGE), we have studied five cell lines: (1) parental SV40-immortalized, non-neoplastic, anchorage-dependent, neonatal prostate epithelial cells (267B1); (2) radiation-treated (2 x 2 Gy), anchorage-independent, non-tumorigenic derivatives (F3-SAC); (3) anchorage-independent, radiation-treated (to a total of 30 Gy) tumorigenic cells derived from F3-SAC (267B1-SXR); (4) malignant cells transformed by exposure of parental 267B1 cells directly to 30 Gy of X-rays (267B1-XR); and (5) v-Ki-*ras* transformed, anchorage-independent, neoplastic transformants (267B1-*ras*).

Materials and Methods

Cell culture

Immortalization of the human neonatal prostate epithelial cell line (267B1) has been described previously (Kaighn *et al.*, 1989). Briefly, these cells were established by transfecting primary neonatal prostate cells with a plasmid containing SV40 early region containing genes. These cells were anchorage-dependent, and non-tumorigenic in nude mice. They were maintained and grown in a medium containing P4-8F (Biological Research Faculty and Facility, Inc., Ijamsville, MD) containing 2% heat-inactivated fetal bovine serum, hydrocortisone 5 μg/ml, penicillin G 100 U/ml, streptomycin 100 μg/ml. Cells were trypsinized for subculturing, pelleted, washed in phosphate buffer saline (PBS) and resuspended in growth medium. Routine mycoplasma testing was carried out.

Irradiations/Tumorigenicity studies

Exponentially dividing cells were exposed to doses of 2.0 Gy fractions of X-rays from a Siemens (Iselin, NJ) 6 MeV linear accelerator as previously described (Kuettel *et al.*, 1996) and subcultured. They were observed daily for morphological changes, and tested for anchorage-independence after every two-successive fractions of X-rays. 1 x 10^6 cells, in 0.2 ml, were injected subcutaneously into the mid-dorsal interscapular region of male immunodeficient 129J adult mice and observed for tumor development (Kuettel *et al.*, 1996). The scheme in Figure 1 shows the origin of the various cells used in the present study. Generation of *ras* transformed prostate epithelial cells (267B1-*ras*) has been described earlier (Parda *et al.*, 1993).

2D-PAGE

Iso-Dalt equipment for 2D-PAGE was from Hoefer Scientific Co. (San Francisco, CA). Electrophoresis of proteins in the total cell lysates was carried out (O'Farrel, 1975) with minor modifications to suit the use of Iso-Dalt procedures. Iso-electric focussing (IEF) gel tubes were made up of 3.5% acrylamide, 9 M urea, 2% ampholines (blended: 1:3 of pH 3.5-10.0 and pH 4.0-8.0), 2% nonidet-40 (NP-40), 0.03% ammonium persulfate, and 0.01% TEMED (N,N,N',N'-tetramethylene diamine). Cell pellets (5 x 10^6 cells) were solubilized in 250 μl of IEF sample buffer {9 M urea, 4% NP-40, 2% ampholines (pH 8-10), and 1% dithiothreitol (DTT)} and stored frozen at -70°C. Samples (20 μl) were focused for approximately 24,000 V-hours and the isotubes transferred to 10% polyacrylamide slab gels for sodium dodecyl sulfate (SDS)-PAGE and run overnight at 100 V at 20°C. The gels were fixed in 40% methanol and 10% acetic acid for at least 2 hours prior to silver staining (Merril *et al.*, 1983). The procedure consisted of treatment of fixed gels in 10% ethanol, 5% acetic acid for 30 minutes, 1 mM DTT for 30 minutes and 0.1% silver nitrate for an additional 30 minutes followed by color development in 3% sodium carbonate/ 0.05% (v/v) formaldehyde. The color development took about 8-10 minutes for the amounts of protein subjected to electrophoresis in the present study, and the developing reaction was stopped by submerging the gels in 5% acetic acid. Stained gels were stored in plastic bags and scanned for computer analysis.

Computer-assisted analyses of protein expression in two-dimensional gels

Quantitation of protein spot intensities was carried out by scanning and digitization of the silver-stained images followed by computer-assisted comparison of the spots of interest in the various cells. In brief, image acquisition was done by a Nikon f2.8 macro lens (Nikon Inc., Melville, NY), while digitization and image analysis were accomplished by a combination of a 3/110 computer (Sun Microsystems, Inc., Mountain View, CA) interfaced to a scanner and digitizer (PS200 power supply, Photometrics Ltd., Tucson, AZ). Data analysis was performed by using the ELSIE-5 software, an updated version of ELSIE-4 described previously (Olson and Miller, 1988). We quantitated TM isoforms 3, 4, and 5 as well as total actin expressed in the various cell lines used in this study. To provide consistent quantitative measurements in the linear range, we selected a few additional spots, the expression of which did not change in the various prostate epithelial cells and accepted silver stain more uniformly.

Confocal microscopy

Cells were cultured overnight on cover slips (60-70% confluence) and fixed in 3.7% formaldehyde containing 0.1% Triton X-100 for 10 minutes. The cell preparations were further permeabilized in 0.5% Triton X-100 for 5 minutes, and washed in phosphate buffered saline. F-actin was labeled using rhodamine-phalloidin (Molecular Probes, Eugene, OR) and the coverslips were examined using a BioRad (Cambridge, MA) MRC 600 laser scanning confocal microscope equipped with a Nikon inverted microscope using a 40x Nikon oil objective with a variable numerical aperture set at 1.1.

Immunofluorescence localization

The various cell samples prepared for confocal microscopy were processed for immunofluorescent localization of actin. The actin cytoskeleton was observed with conventional epifluorescence microscopy using a Zeiss Photoscope II (Carl Zeiss, Inc., Thornwood, NY) with an oil PlanApo 63X/1.4 NA lens.

Results

Generation of prostate epithelial cells at different stages of neoplastic transformation

Detailed knowledge of the molecular events during the multistep process of transformation can be obtained by analyses of cells representing different stages during progression to malignancy. With this in mind, we developed a protocol of irradiating SV40 immortalized prostate epithelial cells and analyzing those that received different total amounts of radiation. The protocol consisted of irradiating cells with successive 2 Gy doses, and after each 2 x 2 Gy treatment, testing them for morphological changes, development of anchorage independence, and tumorigenicity, for these are the criteria of malignant behavior of cells in culture. The SV40 immortalized parental cells (267B1) were anchorage dependent and non-tumorigenic when injected into nude mice. The clone designated F3-SAC shown in Figure 1 was derived from prostate cells that underwent morphological transformation and exhibited anchorage-independence but were non-tumorigenic. These cells represent a transition during the neoplastic process between the events of initiation/promotion and progression. Moreover, the 267B1 cells irradiated with cumulative total doses of 4-12 Gy exhibited no tumorigenicity (Kuettel *et al.*, 1996). Further exposure of these cells (F3-SAC) to additional 2 Gy doses of radiation yielded tumorigenic prostate epithelial cells (267B1-SXR). At the same time, using another protocol, the parental cells were treated with three courses of radiation treatments, each consisting of five consecutive fractions of 2 Gy doses, with no soft agar cloning steps between treatments. This strategy resulted in tumorigenic prostate epithelial cells designated 267B1-XR. Both 267B1-SXR and 267B1-XR cells developed tumors within four weeks when injected into nude mice. Such tumorigenic cells derived from the mice tumors were reestablished in culture (Kuettel *et al.*, 1996) and used for studies described in the present report.

Characterization of the 267B1, F3-SAC and the 267B1-XR cells has been reported previously (Kuettel *et al.*, 1996). Saturation densities of the anchorage-independent and radiation transformed 267B1 cells were 1.7 x 10^5 and 5.8 x 10^5, in comparison to the parental cells (2.5 x 10^4), respectively. Furthermore, the anchorage-independent (F3-SAC) and radiation transformed (267B1-XR) cells grew in soft agar with cloning efficiencies of 0.26 and 0.30, respectively, while the unirradiated cells failed to grow in soft agar.

Organization of Actin Cytoskeleton in 267B1 cells exposed to X-rays

Actin is the principal component of microfilaments. In non-muscle cells, polymerized F-actin and monomeric G-actin are in dynamic equilibrium, and it is the polymerized F-actin that is present in the stress fibers (Garrels and Gibson, 1976; Herman, 1993). All non-tumorigenic cells have highly organized microfilaments with large numbers of stress fibers (Gowing *et al.*, 1984). The expression and organization of actin has been shown to change during cellular transitions such as cell cycle stages, attachment to substratum brought about by manipulation of cultured cells, such as by serum stimulation (Elder *et al.*, 1984), growth factors (Masibay

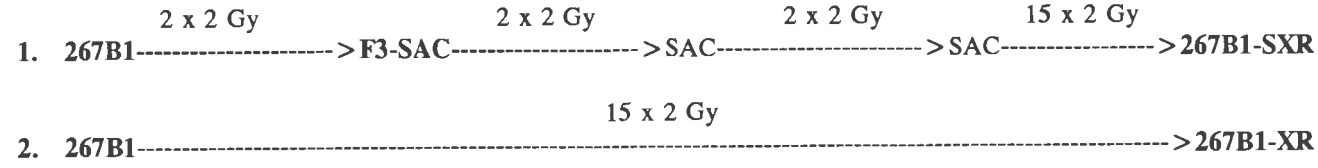

Figure 1. Derivation of radiation-transformed SV40-immortalized human prostate epithelial cells (267B1) by fractionated doses of X-rays: XR represents exposure to ionizing radiation, SAC and S denote soft agar cloning. Immortalized, parental 267B1 cells (Kaighn *et al.*, 1989) were anchorage-dependent and non-tumorigenic in nude mice. F3-SAC cells which received 2 x 2 Gy of X-rays, developed anchorage independent growth. Additional doses of radiation to F3-SAC cells or direct exposure of 267B1 cells to 30 Gy (15 x 2 Gy) as shown resulted in transformed (267B1-XR; 267B1-SXR) cells. The length of the arrows is arbitrary and does not represent the time periods between the radiation treatments.

et al., 1988), or oncogenic transformation (Okamoto-Inoue *et al.*, 1990). It has been suggested that irreversible cytoskeletal rearrangement, ubiquitous in neoplastic cells, may play a causal role in oncogenesis (Leavitt *et al.*, 1985; Maness, 1981; Matsumura *et al.*, 1983; Pollack *et al.*, 1975).

The radiation-treated cells at different stages of neoplastic progression that were used in the present study have certain features in common: their cell size is smaller and are capable of anchorage-independent growth in contrast to the parental 267B1 cells. These features, generated in the *in vitro* model system of SV40-immortalized 267B1 cells, provide suitable criteria to perform studies to establish the earliest time when changes in actin cytoskeleton occur during progression to malignancy.

Using light microscopy, our earlier study demonstrated morphological changes in the F3-SAC cells and the 267B1-XR cells when compared to the parental unirradiated cells (Kuettel *et al.*, 1996). In the present study, we have compared the actin micro-filament organization in three of these radiation-treated prostate epithelial cells using confocal microscopy of rhodamine-phalloidin stained F-actin (Fig. 2). The parental immortalized prostate epithelial cells showed well-developed parallel arrays of stress fibers running the entire length of the cell (panel A). In contrast, the anchorage-independent, non-malignant (F3-SAC) cells exhibited smaller cell size and poor organization of microfilaments (panel B), with their stress fibers being much finer and shorter. 267B1 cells malignantly transformed with v-Ki-*ras* (panel C) exhibited finer stress fibers as in case of the non-tumorigenic, 2 x 2 Gy radiation treated cells (panel B), suggesting that these alterations in the appearance of actin stress fibers are initiated before the development of anchorage independence in these cells.

We next analyzed the actin cytoskeleton in the radiation-induced tumorigenic transformants of these prostate epithelial cells by conventional immuno-fluorescence microscopy. Actin fluorescence in the form of well formed parallel arrays of long stress fibers in the parental 267B1 cells (Fig. 3A) conforms to what is noted in Figure 2A by confocal microscopy. In these parental cells, the stress fibers are predominantly ventral with weak microvilli on the dorsal side and are enclosed in cells with regular outlines. The malignantly transformed prostate epithelial cells, derived from further exposure of F3-SAC cells (267B1-SXR) (Fig. 3C), as well as by direct exposure of the parental cells to 30 Gy (267B1-XR) (Fig. 3D), show short actin fibers in cells that are smaller in size with irregular outlines, as in case of the F3-SAC cells (Fig. 3B). These data further illustrate that radiation-treated cells, either transformed or non-transformed (Figs. 2B, 3B, 3C and 3D), show abundant minute fibers throughout the cytoplasm, but that large bundles of F-actin were not organized. In our radiation treatment protocols during the transformation process, there is at least a 2-3 week period between each successive soft agar cloning steps before an additional 2 x 2 Gy dose is given. The actin stress fiber changes observed in the present study are irreversible and heritable. In contrast, the acute phase response studies of Syrian hamster cells to neutron exposure (Woloschak and Chang-Liu, 1991) and the low dose radiation studies showing endothelial cell retraction (Kantak *et al.*, 1993) report that F-actin changes taking place during the first 10 hour post-irradiation, with their stress fibers reorganizing to their pre-irradiation status within the following 24 hours. It has been noted that stress fibers in normal cells are more stable than short actin filaments of podosomes found in transformed cells (Tanaka *et al.*, 1993). It appears that a less stable cytoskeleton provides cells with a definite growth advantage and increased motility. It is important to note that the parental 267B1 cells receiving two doses of 2 Gy (F3-SAC) showed morphological changes and loss of anchorage-dependent growth, while their actin fiber appearance resembled that of completely transformed tumorigenic cells. These data can be interpreted that acquisition of morphological changes

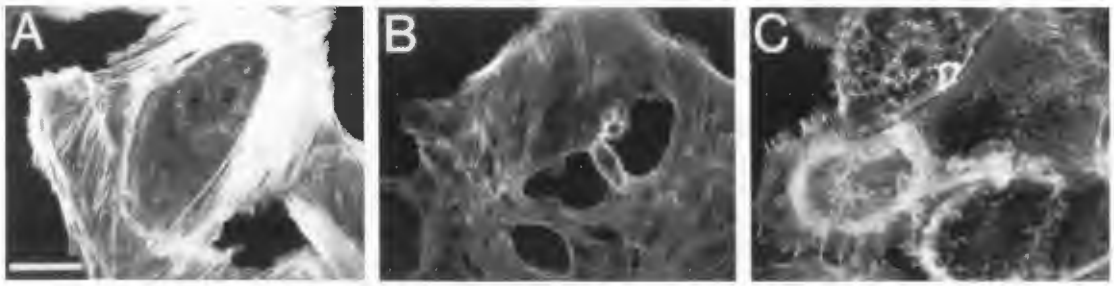

Figure 2. Localization of F-actin in 267B1 cells at various steps in the process of neoplastic transformation using confocal microscopy: (**A**) parental, SV40-immortalized 267B1 cells employed for transformation; (**B**) radiation-treated (2 x 2 Gy), non-neoplastic derivatives (F3-SAC); (**C**) v-Ki-*ras* transformed neoplastic prostate epithelial cells. Bar = 50 μm (the three figures are at same magnification).

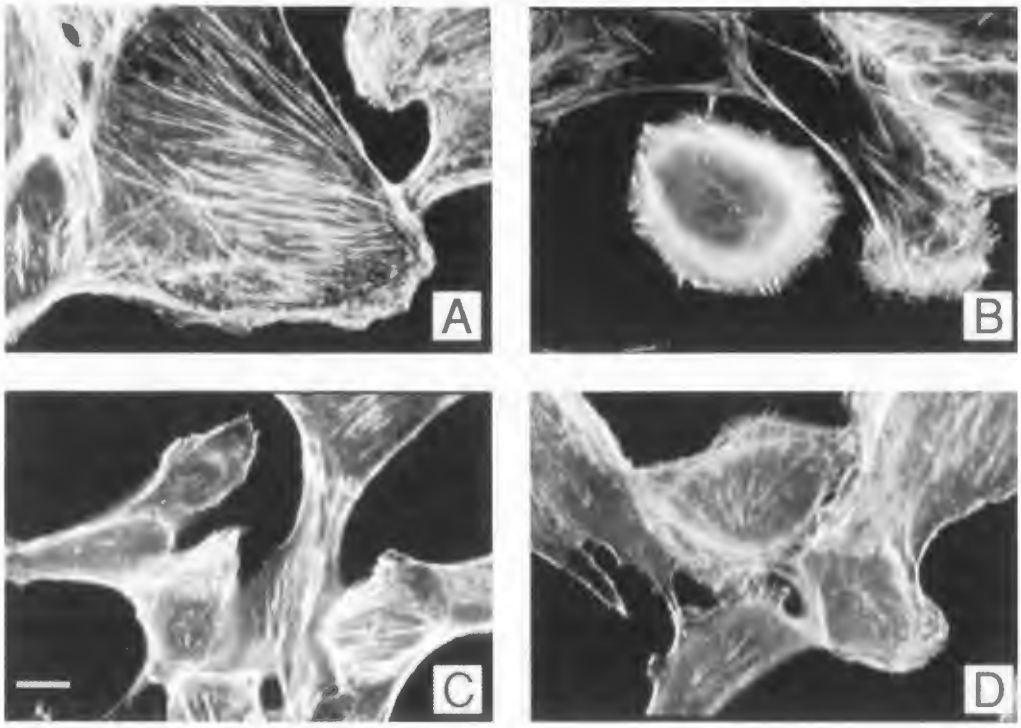

Figure 3. Comparison of actin fiber formation in radiation-transformed tumorigenic prostate epithelial cells by conventional immunofluorescence microscopy: the various cell samples were prepared as in Figure 2, and the actin cytoskeleton observed with conventional epi-fluorescence microscopy using a Zeiss Photoscope II with an oil PlanApo 63X/1.4 NA lens. (**A**) Parental, SV40-immortalized 267B1 cells employed for transformation; (**B**) radiation-treated (2 x 2 Gy), non-neoplastic derivatives (F3-SAC); (**C**) radiation-treated (30 Gy) neoplastic cells derived from F3-SAC (267B1-SXR); (**D**) tumorigenic, transformants following exposure of parental 267B1 cells directly to 30 Gy of X-rays (267B1-XR). Bar = 25 μm (the four figures are at same magnification).

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and the development of anchorage independence are accompanied by the loss of actin microfilament organization, while progression to tumorigenicity takes a longer period of time. It appears that changes in the actin cytoskeleton represent part of the cellular program in the progression toward neoplastic phenotype, whether the transforming agent is ionizing radiation or an oncogene.

Alteration of tropomyosin-isoform expression in the radiation-treated 267B1 cells

Tropomyosin is one of the contractile proteins that makes up the actin micro-filaments and is a potent modulator of the actomyosin system (Lin *et al.*, 1984). TM molecules lie in each of the two grooves along the

Table 1. Relative expression of tropomyosin (TM) isoforms in radiation treated human prostate epithelial cells (267B1).

Cell Type	TM-3	TM-4	TM-5	Actin
A. 267B1	125 ± 29	79 ± 8	72 ± 14	2006 ± 76
B. F3-SAC	74 ± 9	66 ± 13	65 ± 10	1866 ± 162
C. 267B1-SXR	67 ± 7	160 ± 22	162 ± 24	1899 ± 196
D. 267B1-XR	ND	158 ± 8	162 ± 19	2230 ± 295
E. 267B1-*ras*	46 ± 8	186 ± 24	160 ± 9	1976 ± 119

Notes on Table 1: Relative expression levels of protein spots in the various cells were obtained by normalization of spot intensities using the relationship (density of variable spot/density of a constant spot) x 100. The densitometric quantitation of various polypeptide spots is the mean ± S.D. of triplicate gels in each case. ND: not detectable.

length of an actin helix and regulate binding of actin to myosin heads. The suppression of synthesis in TM isoforms has been shown to be common in human fibroblasts transformed with retroviral analogues (Garrels and Franza, 1989; Leavitt *et al.*, 1986; Matsumura *et al.*, 1983). The resulting altered distribution of TMs along the actin fibers predisposes the cells to develop microfilament instability and contributes to the expression of transformed phenotype (Hendricks and Weintraub, 1981; Leonardi *et al.*, 1982).

TMs of normal human epithelial cells have been characterized previously. Cooper *et al.* (1987) and Wirth *et al.* (1992) have reported epithelial cell-specific TM-isoform assignments in two-dimensional gels based on their molecular weights and pIs. In this study, we compared immortalized prostate cells with those malignantly transformed by *ras* oncogene or ionizing radiation for potential transformation-related differences in TM expression. TMs have isoelectric points in the pH range of 4.4-4.6 when resolved by two-dimensional gel analysis as shown in Figure 4. The isoforms (3, 4 and 5) are numbered as indicated in parental 267B1 cells (Fig. 4A). In silver-stained gels, TM isoforms appear to be one of the prominent components of the proteins in these epithelial cells. Comparison of panels A, B, C, D, and E in Figure 4 shows that TM-3 expression is completely lost in the tumor cells derived by direct exposure of 267B1 cells to 30 Gy radiation (panel D). In the radiation-transformed cells obtained from the F3-SAC cells, however, we have noted diminished expression rather than a complete loss (panel C) in comparison to the parental cells (panel A). F3-SAC cells showed an intermediate stage in the transformation process as well as in the changes in TM expression in that their loss of TM-3 expression was only partial as in 267B1-SXR cells (panel C). Another significant feature was the increase in expression of TMs 4 and 5 in both of the tumorigenic, radiation-induced transformants, 267B1-XR and 267B1-SXR (panels C and D), and in the *ras* transformed cells (panel E), while this change was not yet evident in the F3-SAC cells (panel B). Table 1 provides quantitative analyses of the TMs 3, 4, 5 and actin resolved in the same gels. These specific isoforms of TM, differentially expressed in the prostate epithelial system in the present study, appear identical to those reported in cultured epithelial cells, in which the transformed phenotype was induced by viral oncogenes (Matsumura *et al.*, 1983). As is shown in Table 1, parental (267B1) and malignantly transformed (267B1-XR, 267B1-SXR) prostate epithelial cells exhibited identical levels of actin expression. The observed alterations in the cellular concentrations of TMs 3, 4, and 5 in prostate cells at different stages of progression to malignancy may have resulted in the distortion of the ratio of TM relative to actin in the cytoskeleton.

Discussion

During the multistep process of carcinogenesis, cellular changes occur and result in a growth advantage for the neoplastic cells. Characteristic morphological changes have been shown to appear during the earlier passages following carcinogen treatment, while the appearance of criteria such as anchorage independence and tumorigenicity in nude mice may require longer time periods to be established (Pratt *et al.*, 1992; Suzuki *et al.*, 1989).

In this investigation, we performed comparative studies on the molecular organization of the actomyosin system in immortalized prostate epithelial cells and their derivatives, malignantly transformed with X-rays and v-Ki-*ras*-oncogene. We observed that the progress of neoplastic transformation of these prostate epithelial cells is accompanied by poor actin stress fiber formation, as seen by confocal as well as conventional microscopy. The parent cells that express the normal complement of

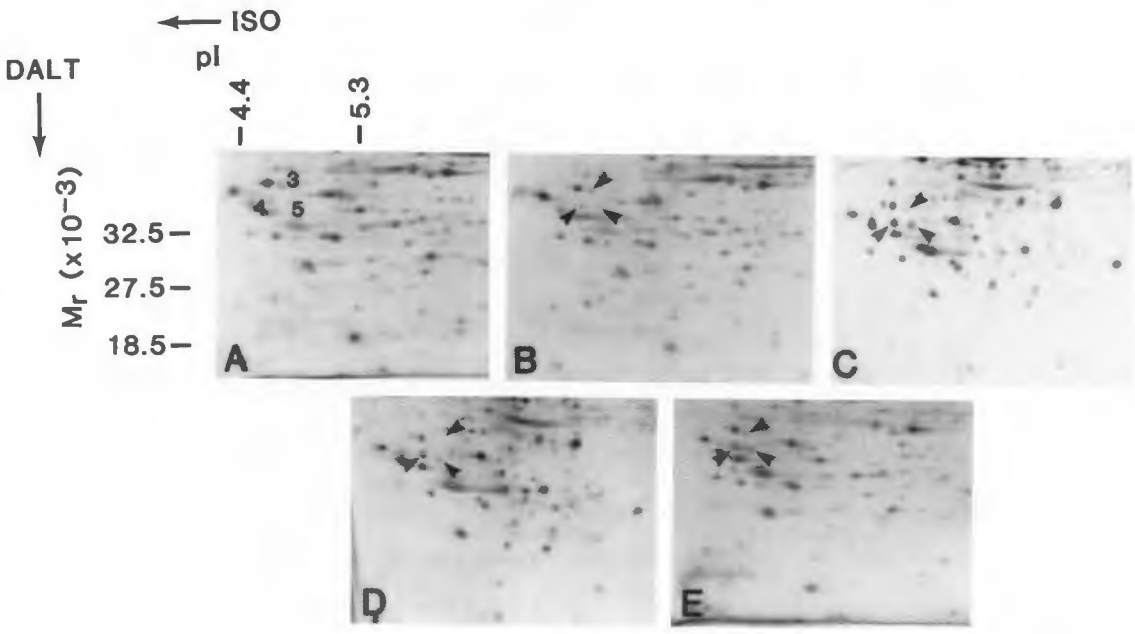

Figure 4. Expression of tropomyosin isoforms upon radiation-induced transformation in human prostate cells: two-dimensionally resolved, silver stained images of total cellular proteins of 267B1 prostate epithelial cells (panel **A**), F3-SAC cells (panel **B**), the radiation-transformants (267B1-SXR; 267B1-XR; panels **C** and **D**) and the *ras* transformed 267B1 cells (panel **E**). TM-isoforms are identified (panel A) based on two-dimensional gel protein profiles (Cooper *et al.*, 1989; Leavitt *et al.*, 1985) and pI and molecular weight (M_r) values of the portions of gels shown are indicated.

actin and TM isoforms maintain anchorage-dependence and non-malignant behavior. The 2 x 2 Gy treated (F3-SAC) cells exhibited altered morphology and poor actin stress fiber organization, as did the malignantly transformed prostate cells (267B1-SXR and 267B1-XR). However, these morphologically transformed prostate epithelial cells, representing an intermediate step during neoplastic progression, did not produce tumors when injected into nude mice, suggesting that they behave as benign tumor cells *in vivo*. These results suggest that change in actin stress fiber organization of the cell appear to be one of the early events in the progression of carcinogenesis brought about by ionizing radiation exposure. However, the present data also indicate that changes in cell size and actin organization alone are not sufficient for the malignant behavior of the transformed cells. The stability of stress fibers depends on the interaction between the four contractile proteins: actin, myosin, caldesmon, and TM and several actin binding proteins such as gelsolin (Cunningham *et al.*, 1992; Vandekerckhove *et al.*, 1990;). The association of cell transformation with the down-regulation of α-actin (Leavitt *et al.*, 1985; Okamoto-Inoue *et al.*, 1990), gelsolin (Vanderkerckhove *et al.*, 1990), higher molecular weight TMs (Matsumura *et al.*, 1983), caldesmon (Koji-Owada *et al.*, 1984), and smooth muscle myosin light chain 2 (Kumar *et al.*, 1989) has been shown. Furthermore, transfection with cDNAs for vinculin (Rodriguez Fernandez *et al.*, 1992), α-actinin (Gluck *et al.*, 1993) and gelsolin (Tanaka *et al.*, 1995) have also been demonstrated to result in suppression of tumorigenicity. Therefore, the underlying changes in microfilament organization upon transformation could be the result of a coordinated behavior of a structural and functional actin organizational unit rather than the effects of ionizing radiation or *ras*-oncogene on a single protein.

While both actin and TMs are part of stress fibers, it is not known whether actin or TM isoforms are the primary regulatory elements in the formation of stress fibers. Recent studies by Prasad *et al.* (1993) support the conclusion that TM suppression is a necessary event for the expression of components of the transformed phenotype, particularly with respect to anchorage-independent growth and tumorigenesis. Such changes in the cellular growth properties correlate closely with neoplastic potential. Studies by Leavitt *et al.* (1985) and Matsumura *et al.* (1983) have noted that the expression of higher molecular weight TM isoforms 1, 2, and 6 (40 kDa, 36.5 kDa, and 41 kDa) is downregulated, while expression of the lower molecular weight isoforms TMs 4 and 5 (32.4 kDa and 32 kDa) is upregulated. The three higher molecular weight TM-isoforms 1, 2 and 6

and TM-3 have been shown to bind actin more tightly than isoforms 4 and 5 (Matsumura and Yamashiro-Matsumura, 1985). Thus, the isoform switching during neoplastic conversion may induce rearrangement of the microfilament system by changing the physico-chemical properties of the stress fibers. In this study, the nature of the TM-isoform alterations, in particular isoforms 3, 4 and 5, was consistent in both of the tumorigenic prostate cell transformants (267B1-SXR and 267B1-XR). In a different model system, using human epidermal keratinocytes, we have shown that these same isoforms of TM are among the differentially expressed proteins in the radiation transformants (Prasad *et al.*, 1994).

The transformation-sensitive changes observed during the progression of carcinogenesis in the 267B1-series of cells also apply to human cells adapted to long term cell culture. The exact situation in tumors growing *in vivo* is not known. Our observations made by fluorescence microscopy and 2D-PAGE support the proposal that the transformed prostate cells adapt to anchorage-independent growth and acquire smaller cell size with short F-actin filaments. It follows that the two identifiable consequences of loss of actin filament organization in these prostate epithelial cells are a change in morphology and the acquisition of tumorigenicity. Several recent reports suggest that cytoskeletal alterations reflect changes in cell adhesion, shape and/or motility (Okamoto-Inoue *et al.*, 1990; Tanaka *et al.*, 1993). The molecular basis of high motility is dictated by the dynamic nature of the changes in the actomyosin system which converts chemical energy into mechanical force. Further studies are required to clarify the physiological function of actin polymerization in the regulation of malignant behavior of cells. The possible alterations in the various actin-binding proteins (Pollard and Cooper, 1986) and the two small GTP-ases, *rho* and *rac* (Norman *et al.*, 1994; Takai *et al.*, 1995), implicated in the control of the actin cytoskeleton, are currently under investigation using the radiation-transformed prostate epithelial cell model system.

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Discussion with Reviewers

H.P. Rodemann: Do the authors have any information about the transformation frequency of neoplastic cells after ionizing radiation? The frequency of tumor cell growth in nude mice should be given.

Reviewer IV: As the phenotype of cells in culture is variable, even for cloned cell lines, a low magnification phase view of the different cultures shown in Figures 2 and 3 would help visualize the differences and the degree of heterogeneity of the different cultures examined.

Authors: The data on transformation frequencies, and the low magnification pictures of the different cultures after exposure to ionizing radiation have been published by Kuettel *et al.* (1996, text reference; 1996b, **Additional References**).

Z. Somosy: Reversible rapid organization of actin cytoskeleton as well as changes in expression of actin gene upon irradiation of cells in culture have been reported earlier (Kantak *et al.*, 1992; Woloschak and Chang-Liu, 1993). The present studies described morphological transformation and parallel changes of actin filament organization upon 2 x 2 Gy irradiation, when the cells did not show malignancy. Please explain this controversy.

H.F. Cantiello: In an effort to validate the contention of this relevant study, perhaps a situation could be envisioned where a chemical such as cytochalsin D could be used to derange the actin cytoskeleton prior to ionizing radiation treatment of otherwise normal cells to be tested for malignancy.

Authors: The frequently described rapid reorganization of cytoskeleton in response to ionizing radiation exposure reported earlier (Kantak *et al.*, 1992; Woloschak and Chang-Liu, 1993) is not comparable to the processes and consequences described in the present study. Their studies have analyzed actin cytoskeletal changes within 12 hours after radiation treatment. The stress fibers in these cells that exhibited an acute radiation response, however, reorganize to their pre-irradiation status within 24 hours.

In the present study, the time difference between the exposure of cells to 2 x 2 Gy doses of ionizing radiation (IR) and analyses of actin fibers is more than two weeks. The procedures after an initial exposure of the prostate epithelial cells to 2 x 2 Gy given on consecutive days, include (a) allowing the cells to grow till they reach confluence; (b) replating on agar plates; (c) picking the soft agar clones followed by subculture to obtain cells for all the experiments performed in this study. Therefore, the changes in the cellular behavior in the IR treated 267B1 cells, that we report, are acquired over a longer time period, they are heritable, and irreversible.

Reviewer IV: A significant mechanistic contribution to the paper might be added by asking whether the level of expression of the tropomyosin isoforms is altered by changes in the level of their expression, or in the stability of the protein?

Authors: Studies are in progress by using cDNA subtraction methods to detect possible differences in transcription in the radiation transformed prostate cells. So

far, TMs have not appeared as candidates. We have not tested the possibility of degradation of usual amounts of TM protein isoforms expressed. However, it is a possibility. We will pursue this in our future studies.

Reviewer IV: Tropomyosin isoforms are known to migrate differently on 2-D gels for each cell type. Therefore, an immunoblot of the 2-D gels is necessary to confirm that the spots marked as isoforms 3, 4, and 5 are indeed the ones claimed by the authors. In addition, quantitative measurements of possible changes in their levels by computerized analysis of the 2-D gels, or by other means, and comparisons made to the appropriate controls are necessary for evaluating the suggested changes in the level of TMs.

G.V. Kulkarni: A major finding of the study were the changes in TM isoform expression. Relative changes in isoform expression can be compared if their levels of expression are standardized to another stable protein whose levels do not change.

Authors: The Sigma (St. Louis, MO) antibody to TM recognized only the 39 kDa spot in the radiation-transformed human epidermal keratinocytes (RHEK) (Prasad *et al.*, 1994). In general, TM isoform nomenclature is followed from the studies of Cooper *et al.* (1987) and Wirth *et al.* (1992). There are no antibodies available that recognize all the isoforms. Further, the isoforms for which we report expression changes are for those that have been the common transformation sensitive candidates and their changes are qualitative and significant. Table 1 provides quantitation of TM isoforms 3, 4, and 5 and actin. We have only made a brief mention of the absence of change in actin expression in transformed cells because this topic has been addressed by earlier studies (Leavitt *et al.*, 1985) as a possible transformation sensitive protein.

G.V. Kulkarni: Confocal and conventional immunofluorescence microscopic data are presented. What distinctly different information does each technique offer?

Authors: Confocal microscopy was initially used with the contention that the actin stress fibers could be visualized in detail. However, our experience with conventional immunomicroscopy revealed identical details and therefore we switched to this technique.

H.F. Cantiello: A positive correlation trait does not necessarily entail a causal relationship, thus the fact that early ionizing radiation induces a derangement of the actin cytoskeleton may not be causal precedent to predict a future course of cell malignancy. It is expected, however, that changes in the length and stability of actin filaments may be directly correlated with cell motility and the ability of epithelial cells to loose anchorage dependency.

Authors: Transformation is a slow process and several endpoints either studied or considered for study are in progress. However, only few of the techniques are sensitive enough to detect changes at every step of the transformation process. In addition to the sensitivities of the methods we can apply, time frames of the sample collection protocols impose additional difficulties. However, the authors do not contend that the observed changes entail invasiveness nor that they represent the causal first step in a linear sequence of events leading the cell to malignancy. The authors claim is that actin cytoskeletal changes and acquisition of anchorage independence occurred sooner than the development of tumorigenic/malignant phenotype in response to IR exposure. Currently, assays for cellular motility are in progress to determine if these cells that exhibit differences in actin stress fiber organization do really exhibit motility differences.

H.F. Cantiello: Are there any cytoskeletal changes in normal cells otherwise exposed to ionizing radiation?

Authors: The protocol of neoplastic transformation that we have employed does not compare well with studies on short term radiation treatment studies. In addition, 2 Gy doses are not damaging enough to induce detectable protein changes in 6-24 hours. It has been our experience that at doses of 6 Gy, protein expression changes have been detected at 6 hours in squamous carcinoma cell studies (Ramsammoj *et al.*, 1996).

G.V. Kulkarni: How does the present study differ from the earlier observations of Matsumura *et al.* (1983) and Leavitt *et al.* (1986)?

Authors: The significance of this study lies in defining the progression events in an *in vitro* human prostate carcinogenesis model system for the first time. The group of cell lines used in the present communication represent defined steps of immortal growth (267B1), anchorage-independent growth (F3-SAC) and tumorigenicity (267B1-XR; 267B1-SXR). Therefore, the characterization of these cells with near isogenic representation of distinct steps in the process of human prostate carcinogenesis is a novel aspect, while confirming the observations of Matsumura *et al.* (1983) in cultured rat cell lines, Leavitt *et al.* (1986) in human fibroblasts and Cooper *et al.* (1987) in mouse and rat fibroblasts. Further, our studies have direct relevance to the development of malignant phenotype in human epithelial systems in general, and prostate carcinogenesis, in particular.

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