ONCOGENE AMPLIFICATION DETECTED BY *IN SITU* HYBRIDIZATION IN RADIATION INDUCED RAT SKIN TUMORS

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ABSTRACT

Oncogene activation may play an important role in radiation induced carcinogenesis. *C-myc* oncogene amplification was detected by *in situ* hybridization in radiation-induced rat skin tumors, including squamous and basal cell carcinomas. *In situ* hybridization was performed with a biotinylated human *c-myc* third exon probe, visualized with an avidin-biotinylated alkaline phosphatase detection system. No *c-myc* oncogene amplification was detected in normal rat skin at very early times after exposure to ionizing radiation, which is consistent with the view that *c-myc* amplification is more likely to be related to carcinogenesis than to normal cell proliferation. The incorporation of tritiated thymidine into the DNA of rat skin cells showed that the proliferation of epidermal cells reached a peak on the seventh day after exposure to ionizing radiation and then decreased. No connection between the proliferation of epidermal cell and *c-myc* oncogene amplification in normal or irradiated rat skin was found. The results indicated that *c-myc* amplification as measured by *in situ* hybridization was correlated with the Southern blot results, but only some of the cancer cells were amplified. The *c-myc* positive cells were distributed randomly within regions of the tumor and exhibited a more uniform nuclear structure in comparison to the more vacuolated *c-myc* negative cells. No *c-myc* signal was detected in unirradiated normal skin or in irradiated skin cells near the tumors. *C-myc* amplification appears to be cell or cell cycle specific within radiation-induced carcinomas.

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INTRODUCTION:

It is widely accepted that exposure to radiation can induce tumors in many organs and species (26). The mechanism responsible for radiation carcinogenesis has been studied and several hypotheses have been proposed (11, 13). The most common one is the " dual radiation action theory ", which states that sublesions are produced depending on the pattern of energy transfers to the cell, and that these sublesions can interact in pairs to produce lesions which in turn determine the observed effect (13). Presumably at least two radiation-induced events are required in tumor development. Ionizing radiation can cause a variety of lesions in DNA, including base modification, single and double strand breaks, sugar damage and DNA-DNA or DNA-protein crosslinking (25). How radiation-induced DNA damages interact each other and initiate a normal cell and subsequently stimulate tumor cell proliferation has become a major issue in understanding radiation carcinogenesis. Carcinogenesis is considered to be a multistage process involving initiation, promotion, and tumor progression (15, 21). The heritable nature of tumor-related-alterations makes it necessary to examine the activity of DNA in different stages of tumor development. Recently the demonstration of specific oncogene activation in radiation-induced tumor cells has been largely responsible for increasing our understanding of radiation carcinogenesis in terms of molecular mechanisms. It has been confirmed that the activation of specific

oncogenes was either directly or indirectly associated with radiation exposure.

Rat skin exposed to ionizing radiation exhibits a variety of tumor types, including squamous carcinomas, basal cell carcinomas, and sebaceous cell tumors (1). The dose-response relationship in rat skin tumors induced by ionizing radiation is well established, and demonstrates that, like the effect of radiation on cell lethality and chromosomal aberrations, the response follows a linear-quadratic pattern (7, 8). Rat skin tumors induced by ionizing radiation provide an excellent model for study of oncogene activation during tumor progression, since tumors are visible early and are available to be excised. A series of biopsies of a growing tumor make possible to the examination of the activation of oncogenes during the development of each individual tumor (3).

Detection of oncogene amplification in rat skin tumors induced by radiation has contributed valuable information for understanding the connection between initiation of carcinogenesis and activation of oncogenes. The results from Southern blot hybridization of the Bam H1 digested tumor DNA with a human *c-myc* third exon probe revealed amplification of the *c-myc* oncogene in 10 of 12 tumors. The *myc* gene was amplified 5 to 20 fold by densitometric analysis. There appears to be tissue specificity in *c-myc* amplification (22, 23). The result of Southern hybridization of tumor DNA from serial biopsies of an individual tumor showed that the *c-myc* amplification was correlated with both the size and growth rate of the tumors. Other information indicated that *c-myc* amplification is more likely a late event rather than a early event in carcinogenesis. It is believed that *c-myc* amplification is related to cell proliferation (14, 20).

The purpose of this thesis is (1) to provide information on *c-myc* amplification of cells from tumors with differing degrees of *c-myc* amplification as shown by Southern

blotting, (2) to examine the *c-myc* amplification in rat skin tissue at early times after exposure to ionizing radiation, (3) to provide information on cell proliferation in rat skin epidermis at early times after exposure to ionizing radiation. *In situ* hybridization was applied in these experiments.

In situ hybridization has provided an efficient tool to localize a specific nucleic acid sequence in tissue sections (9,12,17,18,19,28). The technique is based on the formation of a highly specific hybrid between an appropriately labeled probe of nucleic acid and its complementary sequence in the specimen. This technique can yield both molecular and morphological information about individual tumor cells. More recently the application of biotinylated *c-myc* probe and an avidin-biotinylated alkaline phosphatase detection system has provided the following advantages: rapid detection, improved microscopic resolution, and avoidance of a radiation hazard (2,16,18, 24). The technique of incorporating radiolabeled thymidine into DNA was used to establish epidermal cell proliferation rates.

MATERIALS AND METHODS:

1. Radiation exposure of rats

Male Charles River Sprage Dawley rats (Charies River Laboratory, Kingston, NY), aged 29 days, were exposed to a beam of electrons generated by an AN2500 Van de Graaf ion accelerator (High Voltage Engineering Corporation, Burlington, MA). Rats were anesthetized with 35 mg/kg Nembutal and their backs were shaved. They were then placed inside shielded holding boxes designed to permit the dorsal skin to be pinched up through a 5-cm slot. Radiation was delivered at a dose rate of 7.40 Gy/min, with an energy of 0.8 Mev, for 1 min and 21 second, 2 min and 42 second to give total radiation doses of 10 Gy and 20 Gy respectively. The control rats were prepared for each group. The exposed skin area was marked by using a black marker. After irradiation the rats were returned to regular cages and observed daily for any reactions to ionizing radiation.

2. Preparation of tissue sections:

Following irradiation, rat skin tissue sections were prepared at one, seven, fourteen and twentyone days Rats were anesthetized with 35 mg/kg Nembutal. The exposed skin area was excised from 2 rats for each group. The excised rat skin tissue was fixed either in 10% buffered formalin or in 1:1 acetic acid:alcohol; and then dehydrated in a tissue processor, embedded in paraffin. Sections were cut in 5 μ and placed on the polylysine-coated slides, and then baked for 6 hrs at 60°C.

Coverslips were pretreated for *in situ* hybridization as following: immersed in sigmacote (Sigma Chemical Co. St.Louis, MO) briefly and dried overnight at room temperature.

3. In situ hybridization:

The normal skin tissue sections exposed ionizing radiation and the tumor tissue sections from previous experiments were used for *in situ* hybridization. The slides were dipped twice for 10 min in xylene and once for 10 min in absolute ethanol, then for 5 min sequentially in ethanol-double distilled water (DDW) mixtures as follows: 95%, 80%, 75%, 60%, 30% respectively. Additional steps were as follows: washed in DDW for 5 min and fixed in Carnoy's solution about 5 min; dipped in 0.1% Triton X-100 in phosphate-buffered saline solution (PBS) pH 7.2 for 2 min; washed in PBS and placed in 0.2 N HCI for 20 min; washed in DDW and placed in 2 XSSC at 70°C; washed in DDW for 5 min and treated with proteinase K solution on the surface of tissue section slide; incubated in humid chamber at 37°C. for 15 min; washed in PBS containing 0.2% glycine and dipped in PBS for 1 min; dipped in PBS containing 4% paraformaldehyde at room temperature for 20 min; washed in PBS for 3 successive 5 min; dipped in 95% deionized formamide in 0.1XSSC at 65°C. water bath for 15 min; dipped for 2 min in mixture of ice and 0.2XSSC; dipped for 5 min through a graded ethanol-DDW series containing 50%, 70%, 80%, 90% and 100% ethanol and air dried. Then 20 µl of a prehybridization mixture (200 µg/ml sperm DNA, 100 µg/ml Polyadenylate, 50% (v/v) Deionized formamide, 10% (w/v) Dextran sulfate, 3XSSC, 0.02% BSA, 0.02% Ficoll, 0.02% PVP) was placed on the slides and it was covered with a pretreated coverslip and incubated at 37°C. for 1 hr. The coverslip was removed and the slide was washed in 2XSSC. Then the slide was treated with 10 µl of an hybridization mixture (20 µg/ml biotinylated *c-myc* oncogene probe plus prehybridization), covered with a pretreated coverslip and incubated at 85°C. for 10 min in a humid chamber. The hybridization reaction was kept for 30 min at room temperature after removed from the water bath. Additional steps were: wash in 2XSSC for 5 min and

removed the coverslip; dip for 10 min in 50% deionized formamide in 0.1 XPBS at room temperature; dip for 5 min in 0.05 % Triton X-100 in PBS at room temperature; dip for 20 min in 1XTTBS (0.1 M Tris HCl pH 7.5, 0.1 M NaCl, 2 mM $MgCl_2$, 0.05% Triton X-100, 3% crystalline grade BSA); treat with 100 µl of Vectastain ABC-AP reagent (Vector Laboratories Inc. Burlingame, CA) on section slides and incubated for 30 min. at room temperature; dip in 1XTTBS for 15 min changing the buffer twice. Treat with 100 µl of freshly prepared substrate solution and incubate for 1 hr in the dark at room temperature; wash in TTBS briefly; dip in hematoxylin for 35 sec and washed in DDW; dip in a graded ethanol-DDW series and xylene; mounted for microscopic examination.

4. Injection of tritium thymidine (^3H TdR):

Groups of rats were injected with 1.0 µci/g ^3H thymidine at first, seventh fourteenth, and twenty-first days after exposure to ionizing radiation respectively. After half an hour rats were anesthetized with 35 mg/kg Nembutal and sacrificed by cervical dislocation. The radiation exposed skin tissue was excised and prepared for autoradiography and eosin and hematoxylin staining.

RESULTS:

1. A dry ulceration in a group of rats exposed with 20 Gy of ionizing radiation was observed at day 7 after the expoure.

2. A variety of rat skin tumors were prepared for *in situ* hybridization. Fig.3 (A-D) shows some of the photographs of tumor sections after *in situ* hybridization and staining proces. A-D illustrates a microscopic view of the five biopsy samples from a squamous cell carcinoma which hybridized with a biotinylated *c-myc* third exon probe *in situ*. $T_1(2)$-$T_1(5)$ represent different stages of tumor development. No data on $T_1(1)$ is available . Compared to control the red grains were found in several tumor sections in which biotin labeled *c-myc* oncogene probe was employed. The grains are considered to be a granules of amplified oncogenes and the labeled oncogene probe. The background grain count in normal tissue sections was very low. The density of grains in tumor tissue was dependant on both the type of tumor examined and the stage of tumor progression in five biopsy samples. The results indicate that a certain type of tumor and a certain stage in tumor progression have a high level of grains. The high level of grains on tumor slides implies a high degree of oncogene amplification. All grains observed in section were in tumor cells, not in normal cells. The grains observed were usually found over the nucleus of tumor cells. No grains were found over the cytoplasm or over extracellular space. This pattern of distribution confirms that the grains were the result of binding of oncogene probe to the cellular oncogene. The use of RNAase before hybridization did not change the distribution of grains in tumor section, however the use of DNAase before hybridization eliminated all the grains in the sections, which indicated that the probe hybridized primarily DNA and

not RNA.

Shown in the Table-1 are the results of *in situ* hybridization to the 7 different radiation induced tumors excised from rat skin. Grains were found in tumor sections and the density of grains varied with the tumor type, but correlate generally with the DNA amplification as found by Southern blots. More grains were observed in tumors with high *c-myc* amplification revealed by Southern blotting, including a squamous cell carcinoma (RAD7) in which DNA amplification fold was 20(+). But the grain counts did not always match the result of Southern blotting experiment. More grains were found in the clear cell carcinoma (RAD8) than in poorly differentiated clear cell carcinoma (RAD5); even though the former tumor had a low DNA amplification facter (9x) and the latter one had a high DNA amplification fold (15x). The statistical analysis was based on the counting of grains in tumor sections using the Minitab program (version 7).

Shown in the Table-2 are the results of *in situ* hybridization to five biopsies of squamous cell carcinoma (RAD 106) excised from rat skin at the different times after exposure to radiation. The tumor developed sequentially from $T_1(1)$ to $T_1(5)$, and reflected a progression followed by regression process. But the tumor promotion and induction process may be still limited to investigate based on these samples. Different biotin labeled oncogene probes including *c-myc*, v-H-*ras* and v-K-*ras* were used in the 5 biopsy experiment. By using *c-myc* oncogene as a probe, only samples $T_1(2)$, $T_1(3)$ showed the positive grains, the grains in $T_1(3)$ were more dense than that in $T_1(2)$. There is no data for $T_1(1)$. No grains were observed in any tumor sections without denaturing the DNA in tumor tissue, which indicated that the hybridization between the amplified oncogene and the labeled probe occurred in single strand instead of double strand. Using the biotinylated

rat v-H-*ras* oncogene as a probe, grains were found in tumor sections $T_1(1)$, $T_1(2)$, and $T_1(3)$. The pattern of amplification in tumor development is apparently different for v-H-*ras* from that of *c-myc*. The number of grains was low in $T_1(1)$, high in $T_1(2)$ and medium in $T_1(3)$. But using v-K-*ras* oncogene as a probe no any grains were found in any tumor sections.

Characterization of the tumors revealed that only specific cells types within the tumor sections showed the grains. Under microscopic examination it was found that these cells were relatively small and not vacuolated in compared with the surrounding tumor cells. These cells are presumably involved in the tumor progression.

3. A panel of normal skin tissue sections was prepared as above at first, seventh, fourteenth, twenty-first day after exposure to 0 Gy, 10 Gy or 20 Gy radiation. After *in situ* hybridization with the *c-myc* third exon probe, no *c-myc* amplification signal was found in any of these tissue sections, compared to the positive controls derived from tumor sections. Hyperplasia of epidermis and hair follicles was seen in the sections.

4. Another group of rats was used to assay epidermal cell proliferation after radiation exposure. Rats were irradiated with 0 Gy, 10 Gy or 20 Gy. Following irradiation the rats were injected with 1.0 μci/g ^{3}H thymidine at first, seventh, fourteenth, and twenty-first days. The preparation of tissue sections was the same as above and then autoradiographs. Table 3 shows both the number of total cells per field and the number of labeled cell per field in the epidermal layer counted under the light microscope. The high percentage of labeled cells indicates the high cell proliferation rate at the time of exposure to ^{3}H thymidine. On the first day no significant difference was seen between 0 Gy and 10 Gy, but a large increase of labeled cells at day 7 in 10 Gy group occurred, followed by a

decline at day 14 and day 21. Since day 7 the epidermal layer of skin exposed to 20 Gy of radiation was not found, possibly due to a dry ulceration reaction to the irradiation. The labeling data for 10 Gy and control group are plotted on Fig 1 and Fig 2. The data in Fig 1. indicates the labeling index reached a peak at day 7 and then decreased close to the normal level at day 14 and day 21. The total cell count in Fig 2 shows the same pattern as the labeling index.

DISCUSSION:

Specific oncogene activation in tumor cells has attracted much attention recently. Different approaches have been applied to explore the correlation between specific oncogene activation and tumor development. The radiation-induced rat skin carcinogenesis model has been extensively used to examine oncogene activation. Some rat skin tumors tested were positive in the NIH/3T3 transfection assay, implying activation of the ras oncogene family. Southern analysis of the tumor DNAs revealed evidence for *c-myc* gene amplification with 5- to 20- fold. Also the amplification of *c-myc*, H-*ras* or K-*ras* was found to be associated with an increase in tumor size and *c-myc* amplification was associated with an increase in growth rate. Amplification of *c-myc* was found to occur as a late-stage event in the tumors examined. These results suggest that *myc* and *ras* genes play unique roles in the growth and development of radiation-induced rat skin tumors.

The results here provide evidence for the amplification of *c-myc* in specific cells in several different types of rat skin tumors showing *c-myc* amplification by Southern blotting. The density of grains observed corresponded roughly to the amplification of the oncogene. In five biopsy samples the observation that *c-myc* oncogene amplification was correlated with stages of tumor development was consistent with results from the Southern blotting experiments. There are only small differences in the pattern of *c-myc* oncogene amplification in the different stages of tumor development based on these 2 methods. In Southern hybridization analysis, *c-myc* copy number was the greatest in the second biopsy $T_1(2)$ and decreased in subsequent ones while the grain count was greatest in the third biopsy, $T_1(3)$, by *in situ* hybridization.

A major difference was the finding of amplification of H-*ras* oncogene in five biopsy samples by *in situ* hybridization detected here, but the absence of this finding in Southern blotting experiments. The pattern of H-*ras* amplification was different from that of *c-myc*. The fact that H-*ras* and *c-myc* exhibited different amplification patterns implies that different oncogenes may be activated in different stages of tumor development and may play different roles. No amplification of K-*ras* oncogene was detected in any of the five biopsy samples. It is convincing to believe that *c-myc* amplification is related to radiation-induced carcinogenesis, but it is still difficult to determine whether *c-myc* amplification may be an early event in tumor development. The earliest biopisy sample, $T_1(1)$ was too small to adhere to the slide for the detection of *c-myc* amplification. Tissue samples in earlier stages after exposure to radiation will be required for further elucidation of the role of *c-myc* amplification in radiation carcinogenesis.

The rat epidermis at very early times, such as, seventh day, fourteenth day and twenty-first day, after exposure to ionizing radiation did not show any significant *c-myc* amplification, compared to tumor tissue that served as a positive control. The fact that the proliferation of epidermal cells showed a large increase at day 7 indicates that early proliferation of epidermal cells is likely to be independent of *c-myc* amplification. This evidence is consistent with the view that the *c-myc* amplification is not likely to be involved in early cell proliferation following exposure to ionizing radiation. No connection between epidermal cell proliferation associated with radiation wound regeneration and *c-myc* amplification can be inferred based on these results.

Possibly more significant are results that a morphologically distinct subpopulation of tumor cell exhibited *c-myc* amplification. Results showed that the oncogene amplification

occurred only in specific tumor cells characterized as smaller relatively unvacuolated cells. These cells are probably involved in tumor growth. A pattern of *c-myc* arrangement around the nucleus of tumor cells was found and may be an important clue to oncogene activity. Right now no conclusion can be drawn from this observation, but consideration of this pattern will help our understanding of oncogene action in the future.

The results here confirmed that the application of *in situ* hybridization using biotinylated oncogene probes in tumor sections was a reliable method to investigate oncogene activities in carcinogenesis and made possible the revelation that oncogene activation is cell specific within the developing tumor. The use of tritium labeled oncogene probe in *in situ* hybridization did not show any significant oncogene amplification. The change to using the biotin labeled probe greatly increased the detection of *c-myc* amplification. This also avoided an isotope disposal problem and is relatively rapid in contrast to the long exposure time of the autoradiographic method. Initially the loss of tumor tissue from the slide was a difficult problem due to the high temperature treatment and tiny contact area of tissue on slide. Both adhesion of tumor tissue to the slide and a smooth coverslip are important requirements for a successful experiment using *in situ* hybridization. Polylysine coated slides and siliconized coverslip using Sigmacote have proved to be an efficient way to keep the tumor tissue on the slide.

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TABLE-1.

C-MYC AMPLIFICATION IN DNA FROM RADIATION INDUCED RAT SKIN TUMORS DETECTED BY *IN SITU* HYBRIDIZATION USING A BIOTINYLATED *C-MYC* THIRD EXON PROBE.

No.	Tumor type	DNA amplification (Southern blotting)	Grain count[1] % (*in situ* hybridization)	Mean[2] (*in situ* hybridization)	Adjusted mean[3] (*in situ* hybridization)
RAD1	Poorly differentiated clear cell carcinoma	>5(+)	60%	1.34	2.90
RAD4	Sebaceous carcinoma	15(+)	53%	2.07	3.70
RAD5	Poorly differentiated clear cell carcinoma	15(+)	44%	1.57	4.70
RAD7	Squamous cell carcinoma	20(+)	62%	1.60	2.90
RAD8	Clear cell carcinoma	9(+)	20%	0.70	3.70
RAD9	Sarcoma	1(-)	3%	0.03	0.03
Control			5%	0.05	0.05

1. The number of cell in which at least one grain occurred over the number of total cell counted.
2. The unit of grain mean is grains / cell.
3. The mean adjusted for the existence of two cell populations.

TABLE-2.

ONCOGENE AMPLIFICATION DETECTED BY *IN SITU* HYBRIDIZATION IN FIVE BIOPSY SAMPLES FROM A SQUAMOUS CELL CARCINOMA INDUCED BY IONIZING RADIATION IN RAT SKIN.

Biopsy #	Time from irradiation (week)	Tumor size (cm^3)	Growth rate	Oncogene amplification		
				c-myc	H-*ras*	K-*ras*
1	13	0.2	0.1	ND	+	ND
2	27	2.2	3.6	++	+++	-
3	33	15.0	3.2	+++	++	-
4	39	2.7	-2.2	-	-	-
5	42	1.6	-2.5	-	-	-

The grain was observed under a microscope:
(+): low grain.
(++): intermedium grain.
(+++): high grain.
(-): no grain.
ND: no data.

TABLE-3.

THE PROLIFERATION OF EPIDERMAL CELL FOLLOWING EXPOSURE OF RAT SKIN TO IONIZING RADIATION .

Day	1			7			14			21		
	N*	N**	L.I.	N*	N**	L.I.	N*	N**	L.I.	N*	N**	L.I.
0 Gy	11/43	71/7	0.025	10/49	63/6	0.019	27/30	58/5	0.078	34/99	57/5	0.03
	1/41	60/6	0.002	12/51	74/6	0.019	0/51	54/7	0	15/89	67/7	0.02
			0.014			0.019			0.039			0.025
10 Gy	5/42	69/6	0.01	38/16	64/5	0.19	26/36	69/6	0.063	32/44	58/4	0.05
	1/32	64/7	0.003	34/14	90/5	0.14	31/28	72/6	0.092	27/34	69/6	0.07
			0.007			0.165			0.078			0.06
20 Gy	0/46	65/6	0	NE	NE		NE	NE		NE	NE	
	0/42	55/7	0	NE	NE		NE	NE		NE	NE	

N*: The number of labeled cell per number of field.
N**: The number of total cell per number of field.
L.I.: Lable Index (N^{*}/N^{**}).
NE: No epidermise layer was seen, probably lost due to the dry ulceration.

Fig.1: The labeling index of the epidermis following exposure of rat skin to ionizing radiation.

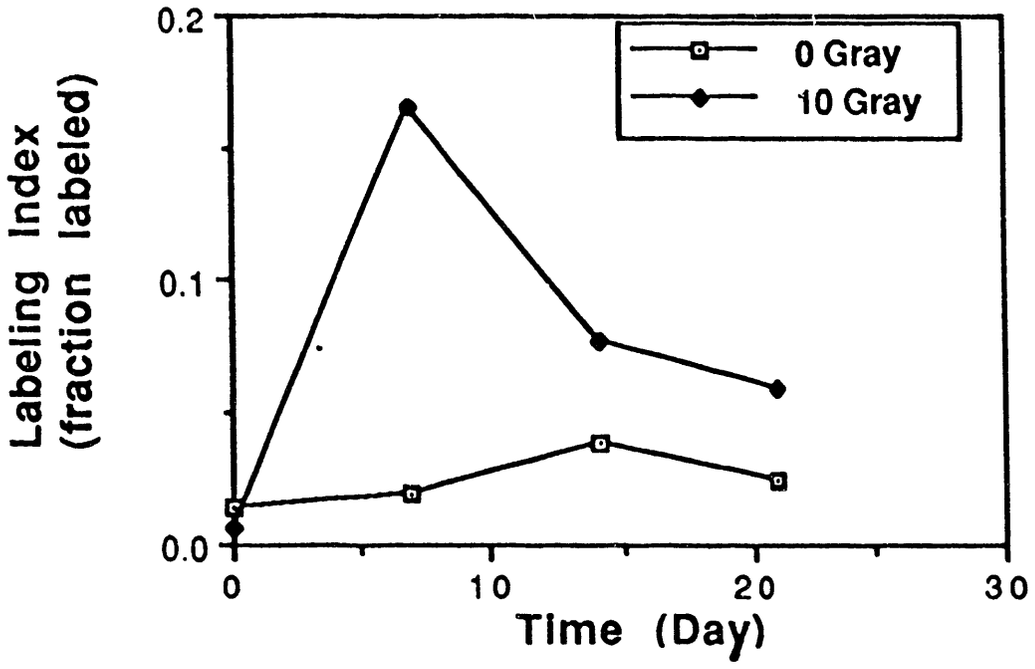


FIG.2: The total cells in the epidermis at various times following exposure of rat skin to ionizing radiation.

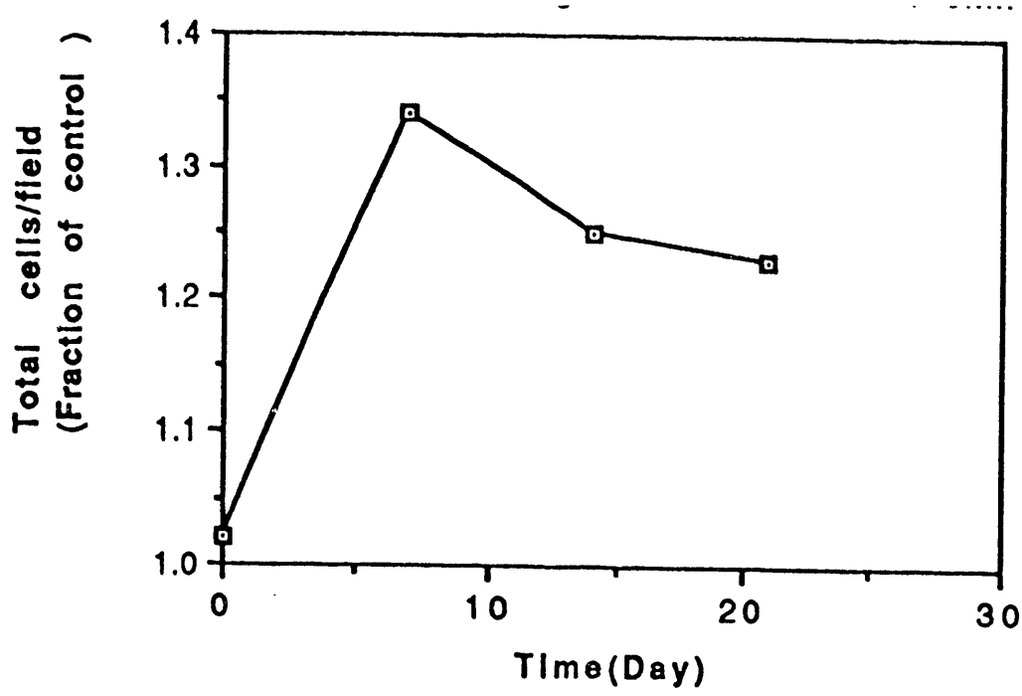
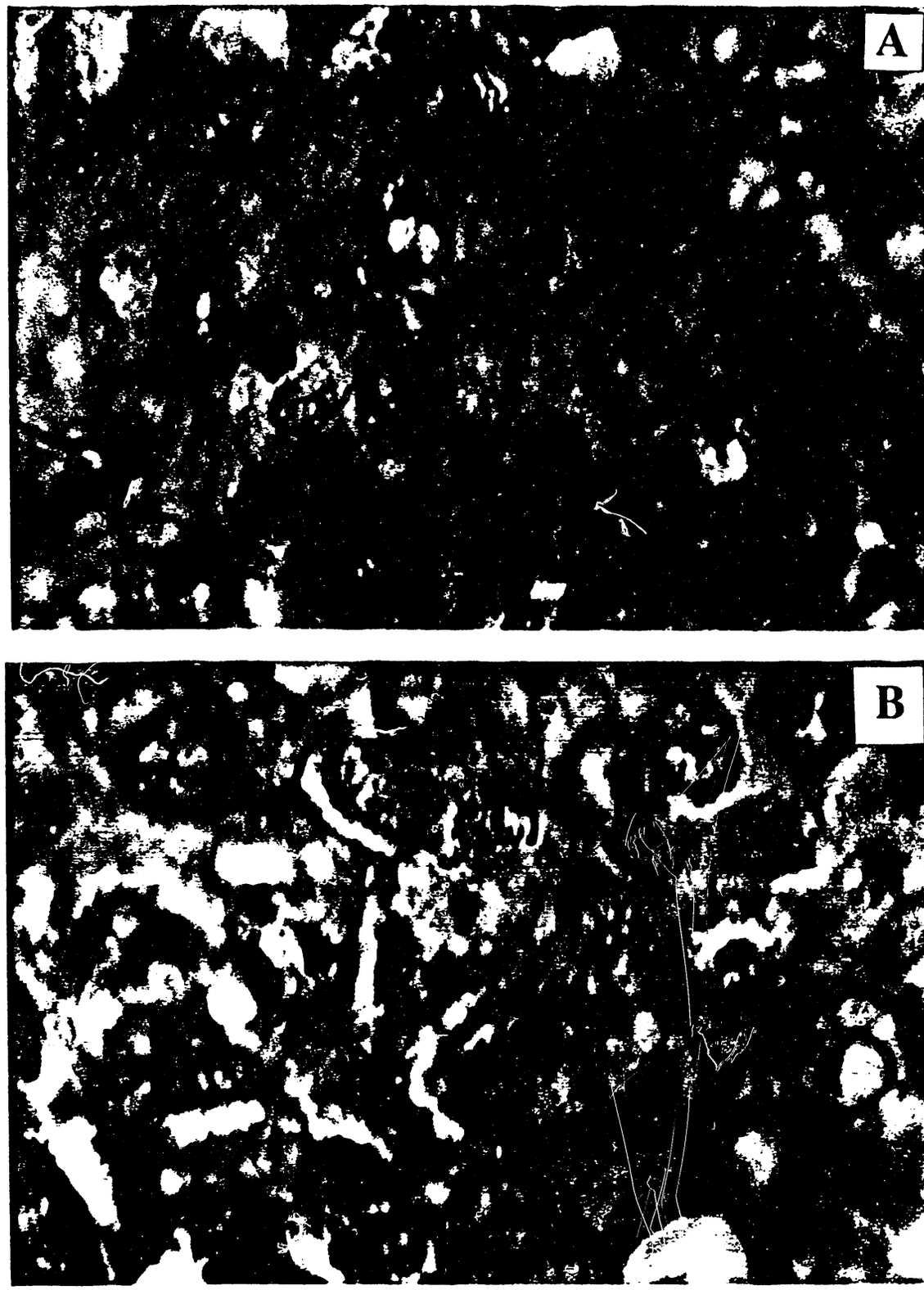
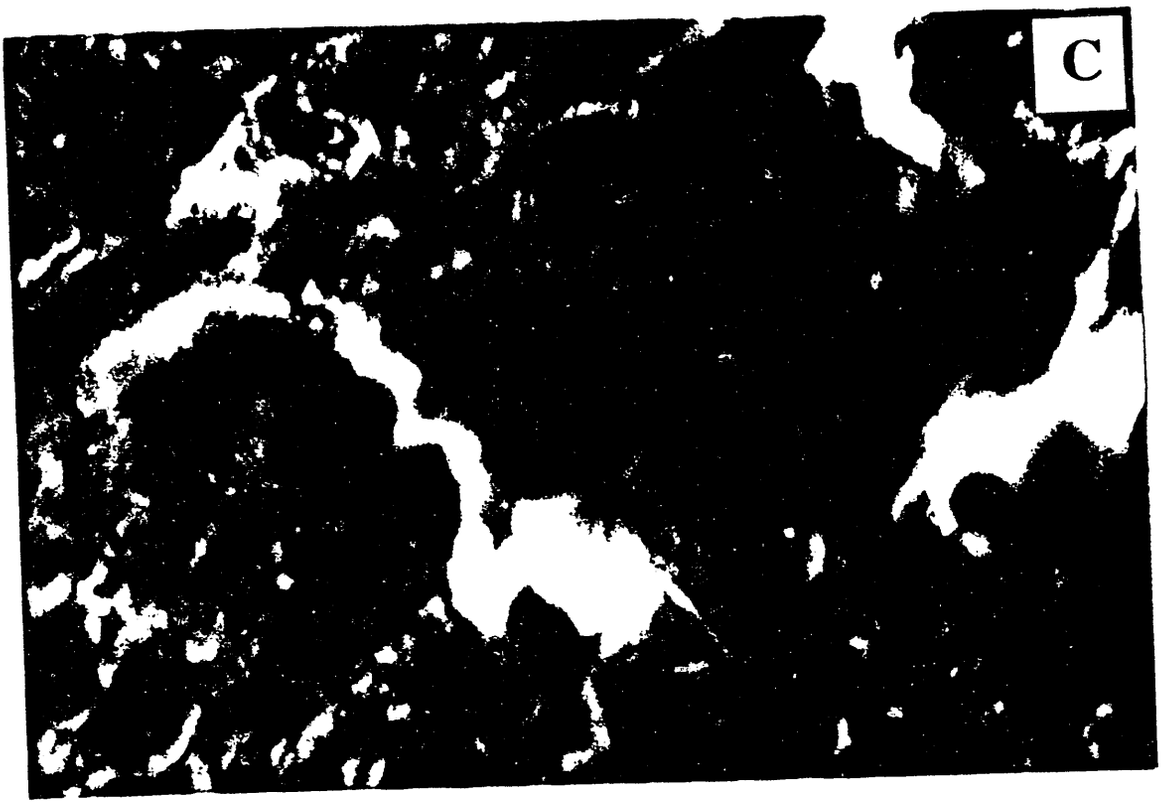
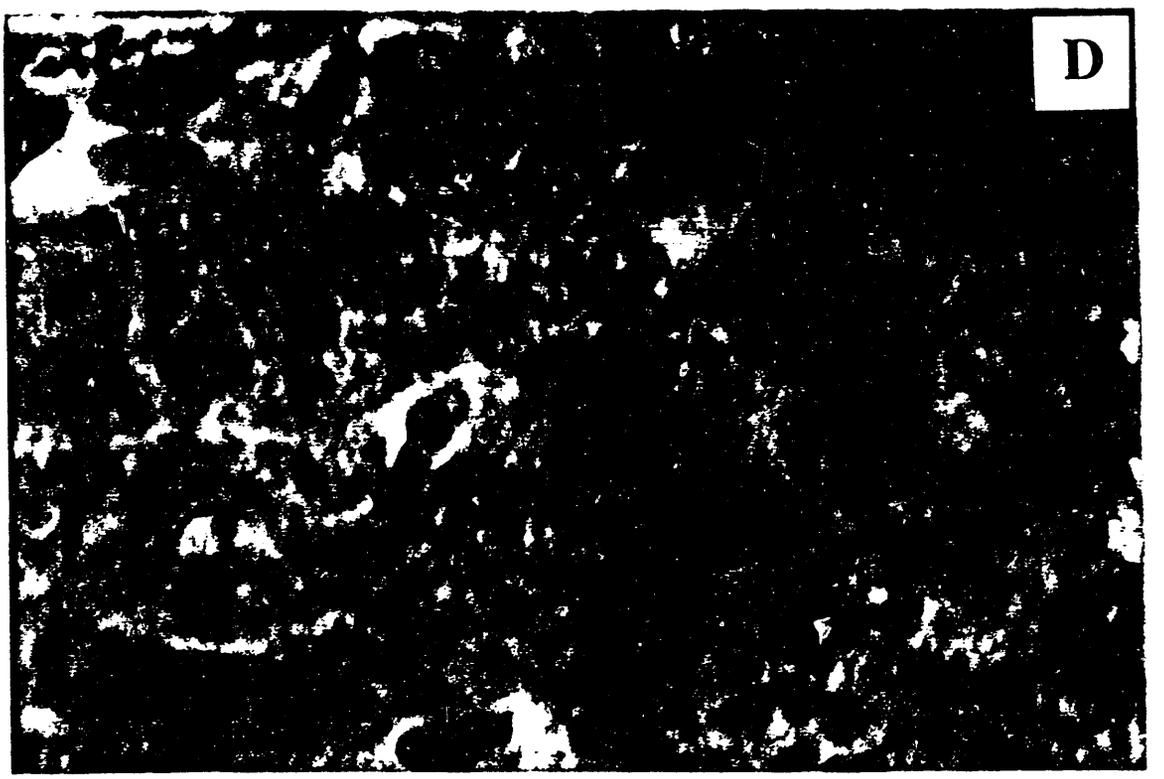


Fig.3: (A-D): Photomicrographs of tissue sections of the five biopsy samples from a squamous cell carcinoma after *in situ* hybridization with a *c-myc* third exon probe. $T_1(2)$-$T_1(5)$ represent different stages of tumor development. A:$T_1(2)$, B:$T_1(3)$, C:$T_1(4)$, D:$T_1(5)$.




C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