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Preferential Induction of c-fos versus c-jun Protooncogene during the Immediate Early Response of Pig Skin to γ-Rays

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Abstract

The involvement of the nuclear protooncogenes c-fos and c-jun in the immediate early response of pig dermis cells was studied after in vivo γ-irradiation. Following high radiation doses (8 to 48 Gy), the two protooncogenes were concomitantly induced, although c-fos induction was preferential. Both inductions were time and dose dependent. Therefore, the early response of the skin to high doses of radiation might involve heterodimeric activator protein 1 composed of c-Fos and c-Jun proteins. Following low radiation doses (0.5 to 2 Gy), c-jun was not induced. By contrast, dramatic c-fos induction was observed after 0.5 Gy, suggesting a specific role for c-fos at low doses.

Introduction

Recent studies have demonstrated that, at the cellular level, ionizing radiation induces an immediate early response, which involves signal transduction pathways and a cascade of genetic events that may contribute to the biological effects of radiation (1).

In this cascade, the involvement of the nuclear protooncogenes jun and fos has been proposed. jun and fos belong to multigene families and are the components of the transcription factor AP-1.3 AP-1 is a DNA binding protein which regulates the transcription of various genes (2). AP-1 is a dimer composed of various Jun and Fos proteins, the dimerization of which occurs through their leucine zipper region. Fos proteins cannot form homodimers and are thus not able to bind DNA on their own. The c-Jun protein can dimerize with itself and then bind as a homodimer to DNA. However, the heterodimer FosJun has an increased affinity for AP-1 sites. These various combinations probably determine the functions of AP-1 and the genes which it regulates. For example, genes responsive to phorbol esters are regulated through the TGACTCA sequence or TPA-responsive element (3). Both Fos and Jun normal cellular proteins possess transforming properties (4).

The involvement of c-jun in the response to radiation was clearly demonstrated in vitro during the immediate response of both normal and transformed cells to high doses of radiation. Thus, leukemia HL-60 cells and diploid foreskin fibroblasts responded to high doses of γ-rays by transcriptional activation of their c-jun gene. This activation was time and dose dependent (5). Although detectable increases in mRNA were already observed with 5 Gy, its level peaked after 50 Gy. Similar c-jun induction was subsequently shown after γ- or X-rays for various other cell types and for both normal and transformed cells (6–8). Consequently, the hypothesis that the transcription factor AP-1 is involved in the immediate early cellular response to radiation (1) is substantially supported by these in vitro results. However, similar results have not yet been reported after in vivo irradiation.

Contrary to the involvement of c-jun in the radiation response, that of c-fos is controversial, although it has been investigated both in vitro and in vivo. Thus, in vitro, treatment of cell cultures with 20 Gy of X-rays did not raise the level of c-fos mRNA in the following cell types: a sarcoma-derived cell line; a histiocytoma-derived cell line; epithelial tumor cells; and diploid foreskin fibroblasts (6, 7). In vivo, neither irradiation of mouse intestine with 50 Gy of JANUS fission-spectrum neutrons (9) nor irradiation of mouse liver, and intestine with 3 Gy of γ-rays (10) resulted in any induction of the c-fos gene during the immediate early response. The only example of c-fos gene induction was found in leukemia HL-60 cells, which responded to high doses of γ-rays by an increase in their c-fos mRNA level. This increase was very similar to that of c-jun in the irradiated HL-60 cells (5).

Overall, these results show that the immediate and early gene responses to irradiations are still poorly understood. Which members of the jun and fos gene families are induced? What is the dose range of the inductions? Are they tissue or cell lines specific responses? Is it possible to extrapolate from in vitro to in vivo? This study aimed at answering some of these questions concerning the immediate early response of skin cells to in vivo irradiation, which, as far as we know, has never yet been studied. For this purpose, we examined the expression of c-fos and c-jun protooncogenes in the dermis of the pig skin removed at various times after γ-radiation doses ranging from 0.5 to 48 Gy. The pig was chosen as an experimental model because it is the reference in radiobiological studies of the skin. The dermis was chosen because previous publications showed that the c-fos gene was not constitutively expressed in that tissue (11) and in order to compare the in vivo response of fibroblasts to the reported in vitro response of cultured fibroblasts. Our results show that both low and high doses of radiation induced preferentially c-fos versus c-jun transcription factor in the pig dermis cells.

Methods

Irradiation. Sixteen Large White pigs were γ-irradiated. They were 5 months old and weighed about 70–80 kg. Permission for this animal experiment was obtained from the Animal Protection Office of the French Ministry for Agriculture and Forestry (Permit 3255).

The irradiation procedure with the 199Ir source has been described previously (12, 13). The 2-cm-diameter collimated source was applied to the skin surface of the flank of anesthetized animals. The dosimetry study was performed using 2 ionization chambers connected to a 2500/3 Ionex dosimeter and with precalibrated lithium fluoride and alanine thermoluminescent dosimeters. For high dose irradiations, the dose rate was 3 Gy/min. Doses of 8, 16, 32, and 48 Gy were delivered to the flank of 10 animals, and 2 animals received an additional dose of 2 Gy. For low dose irradiations, 4 animals received 0.5 and 2 Gy at a dose rate of 0.6 Gy/min.

Clinical studies showed that no change in the skin was observed after a skin surface dose of 16 Gy. After 32 Gy, erythema developed within 8 weeks. After 48 Gy, erythema appeared within 3 days, and dry desquamation of the epidermis developed at the 12th week postirradiation (13).

Tissue Removal. For the high dose experiment, the irradiated skin samples were removed at 2 h (2 pigs), 6 h (6 pigs), 15 h (2 pigs), and 24 h (2 pigs) after irradiation. For the low dose experiment, the samples were removed at 6 h (4 pigs). Control skin samples were taken from the non irradiated flank of each animal. The epidermis was removed and the dermis was directly frozen in...
liquid nitrogen. In addition, the following tissues were removed from one control, nonirradiated animal: ovary; kidney; peripheral lymphocytes; adrenal gland; thymus gland; thyroid gland; liver; and parotid gland. Tissues were surgically removed and directly frozen in liquid nitrogen. Hamster epileptic brain was a generous gift of Jacques Servieres (INRA, Jouy en Josas, France). Epilepsy was induced by tetrazole (14).

RNA Isolation from Tissues. RNA was isolated as described previously (15). Frozen tissues were crushed in liquid nitrogen and placed in 4 M guanidinium isothiocyanate solution, and total RNA was then isolated using the acid-phenol-chloroform method. Total RNA was applied to one oligodeoxynucleotide column (Pharmacia LKB, Uppsala, Sweden) and quantified by monitoring absorbance at 260 nm. Under these conditions, about 50% of the RNA recovered was polyadenylated RNA.

Northern Blot Analysis. For Northern blotting, RNA was separated in 1% agarose gel containing 0.66 M formaldehyde and transferred onto Nytran filters (Schleicher and Schuell). Filters were UV cross-linked. The filters were hybridized to the probes at 65°C overnight. Probes were labeled with [α-32P]-dCTP by random priming (Megaprime DNA labeling kit; Amersham) with a specific activity of 1–2 × 10⁹ cpm/μg. After washing in 0.5 × standard saline-citrate (1 × 0.15 M NaCl, 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate at 60°C, blots were exposed for various times at -70°C using intensifying screens. The following probes were used: a 450-base pair DNA fragment which corresponded to the 4th exon of the human c-fos gene cloned in pSP65 (gift from Dr. C. Lavialle) and was released by restriction endonucleases AccI and EcoRI and subcloned into pUC 18; a 1.4-kilobase complementary DNA fragment, cloned in the SP6 vector (2) encoding part of the trans-activation domain, the DNA-binding domain, and the 3'-untranslated sequences of the c-Jun protein; a 1.6-kilobase probe coding for the 18S fraction of mouse rRNA (16). This probe was used as a control of RNA loading.

On each blot, RNA isolated from hamster epileptic brain was used as a positive control for protooncogene expression (14). Autoradiograms were scanned using a densitometer and analyzed using the Biocom software package. The intensity of fos and jun hybridizations was normalized against 18S fraction expression.

Survival Curve. Skin biopsies were minced and incubated with 0.5% trypsin and 0.25% collagenase. Irradiation was performed on the resulting cell suspensions at doses ranging from 2 to 10 Gy (γ-ray, 0.5 Gy/min). Cells were then seeded at a density of 500 cells/cm² and allowed to grow for 2 weeks. Cultures were then fixed and counted.

Results

High Dose Experiments

c-fos Induction in Pig Dermis Cells. To determine the immediate and early effects of γ-rays on pig skin, the expression of c-fos protooncogene was studied by Northern blotting. c-fos was not constitutively expressed in normal control dermis cells (Fig. 1). γ-Irradiation induced a dramatic induction of its mRNA in the pig dermis cells (Fig. 1; Table 1), inasmuch as the mean maximum induction after 48 Gy was 28 times the control level (range, 12 to 55). Such a response was comparable to that observed in epileptic brain, which is known as a dramatic modification of c-fos expression. This induction was transient. It started 2 h postradiation and was maximal at 6 h (Fig. 2). By 15 and 24 h, the amount of c-fos mRNA had returned to basal level. At 6 h, the threshold dose of induction was 2 Gy.

c-fos gene induction was found for the usual 2.3-kilobase transcript. In addition, radiation-activated tissue exhibited a new transcript with a molecular weight of 1.8 kilobases. Because this transcript has not been described in human cells, we searched for whether it was specific to the species studied. For that purpose, mRNA was isolated from 8 different normal pig tissues and hybridized to the c-fos probe. The major 2.3-kilobase transcript was found constitutively expressed in lymphocytes, ovary, and adrenal gland. The expression of the minor 1.8-kilobase transcript was only found in adrenal gland.

c-jun Induction in Pig Dermis Cells. Fig. 1 shows that c-jun mRNA was constitutively expressed in normal control dermis cells. In the irradiated samples, c-jun expression was induced in a dose-related manner, for both the 3.2-kilobase minor transcript and the 2.7-kilobase major transcript. The kinetics of expression was similar to that of c-fos and exhibited a down-regulation by 15 h (Fig. 2). At 6 h, the threshold dose of induction was 8 Gy. The rise in c-jun induction was much lower than that for c-fos, inasmuch as its maximum mean after 48 Gy was 5 times the control level (range, 2 to 15), compared to 28 times for c-fos. The mean maximal increase was observed following the highest dose of 48 Gy (Table 1). For this dose and at 6 h, all animals exhibited c-jun induction, although with varying intensity.

Low Dose Experiments

Because c-fos induction was already observed after 2 Gy, similar experiments were performed at lower doses, using a lower dose rate (0.6 Gy versus 3 Gy/min). C-jun was not induced after low doses (Fig. 3), while very high increases in c-fos mRNA were detected following 0.5 and 2 Gy, with an amount similar to that observed for the highest doses. Consequently, the c-fos response exhibited a 2-wave pattern, with a first peak at low doses and a second peak for 32–48 Gy.
Clonogenic Assay

The radiosensitivity of dermis pig fibroblasts was determined using the in vitro clonal growth assay. In order to study cells relevant to the in vivo conditions, the assay was performed on primary cultures isolated enzymatically from the tissue. The survival curve for such primary cultures exhibited a $D_0$ of $2.3 \pm 0.4$ (SD) Gy ($n = 2.3$).

Discussion

Little is known about the induction of cellular signaling events and c-jun specific gene expression after cell exposure to radiation. More particularly, these aspects of the response to radiation have not yet been studied in skin. We therefore decided to study the immediate induction of c-jun and c-fos protooncogenes in the $\gamma$-irradiated dermis of the pig skin.

In the normal pig dermis cells, mainly composed of fibroblasts, we found that c-jun was constitutively expressed at a low level. Such a constitutive expression has been described in the epidermis of human skin (11), but not in the dermis. In the pig dermis cells irradiated in vivo, we found a 5-fold maximum induction of c-jun with a threshold dose of 8 Gy. Similar inductions by radiation have been demonstrated in vitro in cultured cells of various types, both normal and transformed cells. Many common features are reported in all the published studies. One of them is the early and transient character of c-jun induction, which is generally observed between 1 and 8 h after irradiation, both in vivo and in vitro, and is subsequently down regulated. Another common feature is that this induction requires high doses of radiation, inasmuch as 15 to 20 Gy are generally needed to obtain significant increases. In addition, the results obtained on transformed and tumor cells show that cell transformation does not alter the induction of the c-jun gene during the immediate response. All these observations suggest that the involvement of the transcription factor AP-1, either as a c-Jun homodimer or as a heterodimer composed of c-Jun bound to another regulatory protein, is probably a general process characterizing the early cellular response to high doses of radiation.

As regards the c-fos protooncogene, we found that it was not constitutively expressed in the normal pig dermis cells, as described by studies on normal human dermis (11). After high dose irradiation, we found dramatic induction of c-fos in pig dermis cells. The present results differ substantially from those reported in previous studies of the radiation response. While most authors found no induction of this gene after irradiation of various cell types, we demonstrated here that, in the dermis, c-fos is induced concomitantly with c-jun following high radiation doses. Therefore, the early response of the skin to high doses of radiation might involve heterodimeric AP-1 composed of c-Fos and c-Jun proteins.

Differences concerning the induction of c-fos may be due to tissue type or cell type specificities (17). Tissue-specific transcription factor activities may be attributable to the use of different members of the Jun and Fos families. The Fos family comprises c-Fos, Fos-B, and FRA1 proteins, and the Jun family, c-Jun, Jun-B, and Jun-D. Various combinations of protein dimerization might correspond to various capacities to bind promoter sequences and therefore to different effects of the transcription factor AP-1.

Differences in induction may also be attributable to differences between in vivo and in vitro responses to radiation. Hallahan et al. (7)
found no induction in normal fibroblasts irradiated in vitro with high doses. In the present study, we mainly studied the response of normal fibroblasts irradiated in vivo, because fibroblasts are the major cellular component of the dermis of the skin, although we cannot exclude that the endothelial cells of the dermis capillaries may also take part in the cellular response. Such a difference between in vivo and in vitro responses to radiation was previously observed for metallothionein expression after X-ray exposure in the mouse liver cells (18).

Furthermore, as regards the induction of c-fos, we found that it occurred following much smaller doses of radiation than c-jun (threshold dose, 0.5 Gy versus 8 Gy), and that the response exhibited a 2-wave pattern, with a first peak of c-fos mRNA after 0.5 and 2 Gy. The induction of the c-fos protooncogene after 2 Gy is of particular clinical interest, because this is the dose per fraction generally delivered in radiotherapeutic treatment of tumors, and consequently a dose which may be absorbed by normal tissues around the tumor.

This preferential induction of the low doses suggests many questions about the mechanisms of action of the Fos protein. It is known that this protein is not able to bind DNA on its own and that as a transcription factor requires binding to other regulatory proteins. Consequently, the three alternative dimerization processes might explain our results for low doses: (a) the action of c-Fos might be dependent on pre-existing c-Jun; (b) Fos might dimerize with other members of the Jun family; (c) it is possible that, at low doses, c-Fos dimerizes with other unknown factors. New experiments are currently being designed in our laboratory to test these hypotheses.

In irradiated pig skin, high doses of ionizing radiation induce damages such as cell death and tissue necrosis, followed by repair processes and resulting in skin fibrosis or retraction (13). The concomitant induction of c-fos and c-jun might therefore regulate the genes involved in such damage. As we recently described that transforming growth factor B, plays a key role in wound healing of irradiated skin (15), these transcription factors might activate the transforming growth factor B, promoter via its AP-1-like sequence.

As regards lower doses, the localized irradiation that we performed results in no clinical lesion under a dose of 16 Gy, and the survival curve of pig dermis fibroblasts in primary culture allowed a D0 determination of 2.3 Gy. Our results suggest that the induction of c-fos alone at moderate and low doses may be involved in the mutagenic effects of radiation and in the repair processes triggered by the cell to prevent them rather than with cell death. The induction of c-fos with doses of 0.5 Gy might be related to the adaptive response described by several authors after ionizing radiation (19).

In summary, the present results demonstrate that the c-fos gene was preferentially induced following in vivo irradiation of pig dermis cells. At high doses of radiation (8 to 48 Gy), both c-jun and c-fos protooncogenes were induced, suggesting the involvement of heterodimeric c-Fos and c-Jun AP-1 protein in the immediate radiation response. At doses smaller than 8 Gy, only c-fos was induced. Therefore, c-fos might exert specific action after low doses of γ-irradiation.

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References

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