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Protein Kinase C-β Is Induced In Ionizing Irradiation Induced Pigmentation

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Cutaneous hyperpigmentation is a well-known consequence of both acute and chronic X-irradiation, although the molecular mechanisms involved are not well understood. Recently, protein kinase C-β (PKC-β) was shown to activate tyrosinase, a key and the rate-limiting enzyme in melanogenesis [1]. In this study, we have investigated its role in mediating ionizing radiation-induced pigmentation by exposing cultured human melanocytes to X-irradiation. Increased tyrosinase activity after the 4 Gys exposure was observed within 48 hrs and total melanin content doubled after 7 days. Interestingly, tyrosinase mRNA level was not affected by X-irradiation. However, there was a 2-3 fold increase in PKC-β mRNA after 48 hours of irradiation, coinciding with the increase in tyrosinase activity. This induction was not due to non-specific heat generated during the irradiation because when melanocytes were incubated at 40°C, there was no induction of PKC-β mRNA. Taken together, these data suggest that X-irradiation induces cutaneous hyperpigmentation, at least in part, by up-regulating the level of PKC-β.

Key Words: X-irradiation, PKC-β, tyrosinase, pigmentation

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INTRODUCTION

Cutaneous hyperpigmentation is a well-known consequence of both acute and chronic X-irradiation. Histologic features of the response include pigment-laden macrophages in the dermis and increased epidermal melanin content and a heightened DOPA oxidase reaction in epidermal melanocytes [2]. Furthermore, in guinea pigs, repeated exposures to the relatively low dose 5 Gy resulted in an increased amount of melanin throughout the epidermis, as well as an increased number of melanocytes in the basal layer, in addition to pigment incontinence [3]. A single exposure to 10 - 150 Gy increased tyrosinase activity in cultured mouse melanoma cells [4].

Several mechanisms through which ionizing radiation might increase melanin production have been proposed. Because X-irradiation increases the permeability of lysosomal, mitochondrial, microsomal and erythrocyte membranes [5-7], suggestions were made that X-irradiation increases tyrosinase activity by increasing permeability of melanosomal membrane such that melanin precursors, mainly tyrosine, can easily be transported [4].

Protein kinase C (PKC) is a major second messenger implicated in a wide range of functions including cellular proliferation, differentiation and transformation [8, 9]. PKC was also shown to be affected by X-irradiation in a number of different cell types [10, 11]. X-irradiation was shown to activate PKC [10], and protooncogenes such as c-jun and c-fos are up-regulated by X-irradiation through PKC-dependent pathway [11].

PKC represents a multigene family with eleven different isoforms identified to date [9]. These PKC isoforms are believed to subserve specific biological functions and recently, the β isoform of PKC has been shown to up-regulate human melanogenesis by activating tyrosinase in cultured human melanocytes [1]. In this study we have investigated whether cutaneous hyperpigmentation induced by ionizing irradiation is mediated through the PKC-dependent pathway.

MATERIALS AND METHODS

Cells and Media. Neonatal foreskins obtained within 2 hours of elective circumcision were used to culture human melanocytes in Medium 199 (Gibco, Grand Island, NY) containing 5~10% fetal bovine serum as previously described [1].

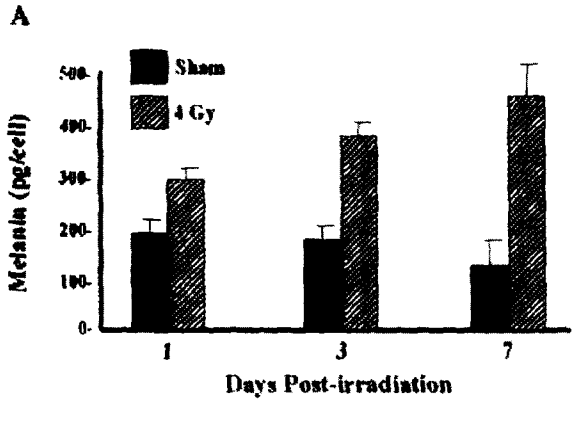
X-irradiation. A 4 MeV linear accelerator (Varian Linac), with a dose rate of 200 cGy/min was used as the x-ray source. Standard calibration methodologies was used as previously described [3]. Cells were irradiated in their culture media and the dose of 4 Gy was selected because it is the routinely used dose in the experimental studies [3]. Control sham-irradiated samples were handled identically.

RESULTS

Pigmentation response to X-irradiation. To examine effect of X-irradiation on melanogenesis, initially the total melanin content of X-irradiated cells was compared to that of sham-irradiated cells. Total melanin content of the two groups remained similar through 24 hrs [Figure 1A]. However, by 3 days post X-irradiation, there was nearly a doubling of total melanin content and at 7 days, total melanin content increased ≥ 3 fold in X-irradiated cells.

To further characterize the effect of X-irradiation on pigmentation, we examined activity of tyrosinase, the rate-limiting enzyme in melanogenesis. 24 hours after X-irradiation, there was no change in tyrosinase activity [Figure 1B]. However, tyrosinase activity increased 60% within 2 days after X-irradiation, while sham irradiated cells showed no change [Figure 1B].

Tyrosinase mRNA level is unaffected by X-irradiation. It has been suggested that the increase in tyrosinase activity after treatment with such agents as α-melanocyte stimulating hormone involves increased level of tyrosinase mRNA and/or protein [14-16]. To explore the mechanism through which X-irradiation increases tyrosinase activity, we therefore examined the level of tyrosinase mRNA. Exponentially growing melanocytes were exposed to a single 4 Gys dose of X-irradiation and then harvested for total RNA at 24 hours and 48 hours. The level of tyrosinase mRNA remain unchanged up to 48 hours after irradiation [Figure 2], suggesting that the increased tyrosinase activity after X-irradiation is due to changes at the post-translational level.



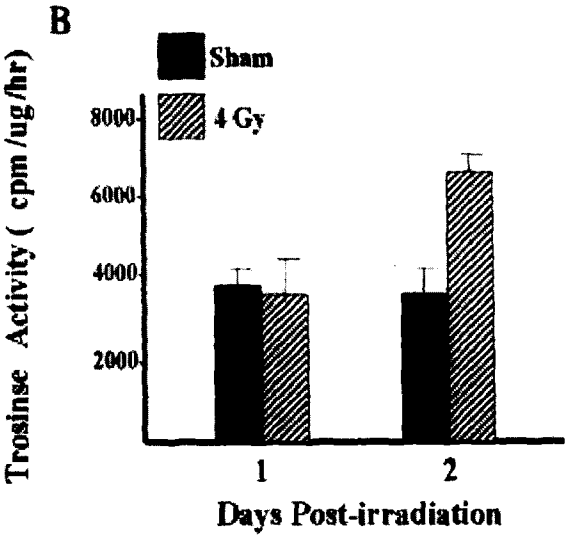


Figure 1: Total melanin content and tyrosinase activity is up-regulated by X-irradiation
(A) A paired culture of melanocytes were either sham or X-irradiated and total melanin level was determined as described [12] on the days indicated. Total of 4 donors was examined and a representative donor is presented.
(B) A paired culture of melanocytes were either sham or X-irradiated and total tyrosinase activity was determined as described [13].

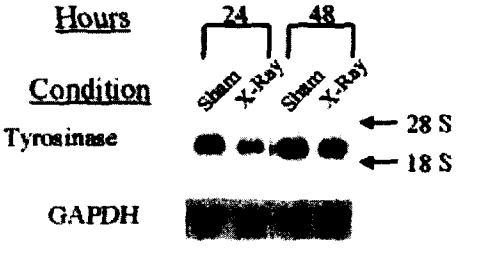


Figure 2: Tyrosinase mRNA is unaffected by X-irradiation: A paired culture of melanocytes was either sham or X-irradiated and harvested for total RNA at each time point. Northern blot analysis was performed as described [1]. GAPDH was used to determine equal loading of RNA in each lane.

PKC-β mRNA is induced by X-irradiation. It was shown by our laboratory that PKC-β specifically regulates human pigmentation by activating tyrosinase through phosphorylation [1]. To test the hypothesis that X-irradiation increases the level of PKC-β, leading the activation of tyrosinase, the level of PKC-β mRNA was examined before and after X-irradiation. Subconfluent human melanocytes were X-irradiated with a single dose of 4 Gys and cells were harvested for total RNA. PKC-β mRNA was induced within 2 hrs of X-irradiation, returned to the basal level at 24 hours, but induced again at 48 hours [Figure 3]. The X-

irradiation-induced increase in tyrosinase activity coincided with the increases in the level of PKC-β mRNA. To exclude the possibility that the PKC-β mRNA induction was due to the heat generated during the X-irradiation, possible induction of PKC-β by heat was explored. Subconfluent melanocytes were placed in the incubator with the temperature at 40^0 C for the indicated amount of time. Heat alone did not induce PKC-β mRNA [Figure 4]. Probing the same blot with heat shock protein 70 showed induction of heat shock protein at 48 hours.

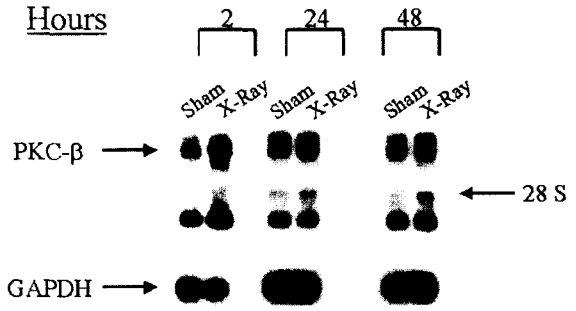


Figure 3: PKC-β mRNA is up-regulated by X-irradiation: A paired culture of melanocytes was either sham or X-irradiated and total RNA was analyzed for PKC-β.

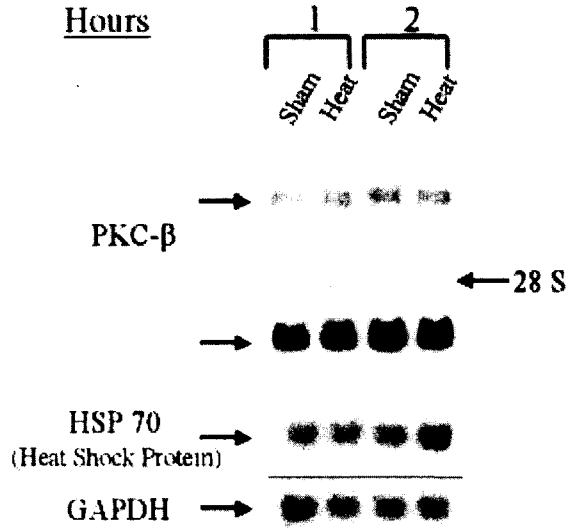


Figure 4: PKC-β mRNA is unaffected by X-irradiation: A paired culture of melanocytes was either sham or heat-induced and RNA was harvest for the level of PKC-β and Hsp 70 mRNAs.

DISCUSSION

Clinical hyperpigmentation involves a complex pathway resulting in an increase in total epidermal melanin content. While ultraviolet light (UV) is the best characterized physiological inducer of pigmentation [17], other types of radiation, such as X-irradiation, also induce pigmentation [2, 3, 18]. Recent work has provided some insight into the initial photoreceptive events underlying the tanning response to UV [19] and radiomimetic chemicals that link X-rays produce single strand DNA breaks [20]. Our results show that with X-irradiation at 4 Gy, a routinely used experimental dose, increases melanin content and tyrosinase activity of human melanocytes.

Increased tyrosinase activity in the absence of increased tyrosinase mRNA level indicates that the regulation of pigmentation by X-irradiation is at the posttranslational level. Such lack of correlation between tyrosinase mRNA and total melanin content and tyrosinase activity has been noted previously [21] without, however, identifying the mechanism by which tyrosinase activity is increased at the posttranslational level. The increased PKC-β expression following in the present study offers one explanation, in that PKC-β was previously shown to activate tyrosinase in human melanocytes [1].

Under physiologic conditions, PKC is activated by diacylglycerol cleaved from lipids in the cellular membrane or membrane of subcellular organelles [9]. It was reported that X-irradiation can disturb membranes [5-7] which can lead to the generation of diacylglycerol. In mouse keratinocytes, UV was reported to generate diacylglycerol [22]. Conceptually, such generation of diacylglycerol can specifically activate PKC-β, which then will lead to the activation of tyrosinase. In fact, in other cells types X-irradiation was shown to up-regulate the protooncogenes c-jun and c-fos known to be regulated through the PKC-dependent pathway [10]. Treatment with PKC inhibitors blocks induction of c-jun and c-fos by X-irradiation [10], further suggesting that X-irradiation is capable of activating the PKC-dependent pathway. However, while generation of diacylglycerol by the interaction between growth factors and their cellular receptors occurs within minutes [9], generation of diacylglycerol by radiation takes hours to days [22]. This may be attributed due to the fact that while activation of receptors specifically activates phospholipase C, which will than cleave phospholipid on the membrane to generate diacylglycerol very efficiently, the generation of diacylglycerol by irradiation may be a non-enzymatic reaction, thus inefficient and requiring longer length of time. To date, it has not been determined whether ionizing radiation can activate phospholipase C.

We conclude that at least the early portion of the pigmentary response to ionizing irradiation is mediated by PKC-β through increased phosphorylation and hence, activation of existing tyrosinase. Our experiments do not exclude a later contribution from enhanced tyrosinase gene transcription, or exposure at 48 - 96 hrs to radiomimetic chemicals [20]. These data expand our understanding of the complex mechanisms underlying clinical pigmentation responses.

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