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Interleukin 6 mediated differentiation and rescue of cell redox in PC12 cells exposed to ionizing radiation

Kazuhiro Abeyama[a], Kazunori Kawano[b], Toshihiro Nakajima[a], Ikuko Takasaki[a], Isao Kitajima[a], Ikuro Maruyama[a,*]

[a]*Department of Laboratory Medicine, Faculty of Medicine, Kagoshima University, Kagoshima City, Kagoshima 890, Japan*
[b]*Department of Oral Radiology, Kagoshima University Dental School, 8-35-1 Sakuragaoka, Kagoshima City, Kagoshima 890, Japan*

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Abstract The differentiation of PC12 cells to a neuron-like morphology was induced by ionizing radiation in the presence of serum. This effect was detectable at 5 grays (Gy) and reached a maximum at 10–20 Gy. Increases in the DNA binding activity of nuclear factor κB (NF-κB) and increased Interleukin 6 (IL-6) mRNA levels were observed at a dose of 15 Gy. Neutralization of supernatant IL-6 by the addition of anti-IL-6 antibody inhibited the neuronal differentiation and decreased cellular redox. Ionizing radiation and serum may act synergistically as neurotropic factors.

Key words: Ionizing radiation; Differentiation; Interleukin 6; Nuclear factor κB; Cell redox

1. Introduction

The rat pheochromocytoma cell line PC12 can extend neurites and differentiate to neuron-like cells in response to nerve growth factor (NGF), and this has been used widely as an in vitro model for studying neuronal differentiation [1]. Recent reports have demonstrated that IL-6 also can induce differentiation in PC12 cells [2]. Several studies have demonstrated that ionizing radiation is associated with the increased expression of cytokines [3–5], including IL-6 [5], through NF-κB activation [5,6]. NF-κB recognizes and binds an 11-bp DNA sequence present in the κ immunoglobulin light chain gene enhancer [7].

The present study demonstrates that ionizing radiation is associated with induction of NF-κB activation and IL-6 production, which induce neuronal differentiation and rescue decreases of the cellular redox system.

2. Materials and methods

2.1. PC12 cell culture and radiation treatment

PC12 cells were routinely passaged in plastic Corning plastic tissue culture dishes in DMEM supplemented with 10% fetal bovine serum and 5% horse serum. Cells were cultured in 35 mm Corning culture dishes for measurement of neurite outgrowth and in 96-well microplates (Coster) for MTT reduction at 30% confluence in Dulbecco's modified Eagle medium (DMEM) supplemented with 0.5% heat inactive fetal bovine serum (FBS) for 24 h. After the culture medium had been replaced by DMEM supplemented with an appropriate concentration of heat inactivated FBS, cells were exposed to ionizing radiation in an X-ray generating system (MBR 1505-R, Hitachi Co. Chiba, Japan).

*Corresponding author. Fax: (81) (992) 75 2629.

2.2. Measurement of neurite outgrowth

PC12 cell differentiation was determined by scoring for neurite outgrowth 24 h following exposure to radiation. Cells possesing one or more neurites, with a length of greater than twice the diameter of the cell body, were scored as positive.

2.3. MTT/Crystal violet cellular reduction assay

Measurement of the cellular reduction was carried out essentially as described previously [8]. Twenty-four hours after exposure to ionizing radiation, MTT was added to a final concentration of 0.5 mg/ml,and incubation was continued for 4 h. Cell lysis buffer (100 μl per well : 20% SDS/50% *N,N*-dimethylformamide, pH 4.7) was then added, and following mixing, colorimetric determination of formazan formation as MTT reduction was made by absorbance at 570 nm. Crystal violet staining to determine the viable cell number was performed as previously described [9]. Twenty-four hours after exposure to ionizing radiation, 100 μl 25% methanol containing 0.5% Crystal violet were added and washed with phosphate-buffered saline and solublized in 10% SDS and then read at the absorbance of 540 nm.

2.4. Detection of IL-6 mRNA using reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR analysis was performed as described elsewhere [10]. Total RNA from the harvested cells exposed to various dose of ionizing radiation was isolated by the acid guanidine thiocyanate/phenol chloroform method [11]. One μg of total RNA was converted to cDNA by reverse transcriptase (Superscript, Gibco. Ltd.). The amplification procedure consisted of 30 cycles (95°C, 30 s; 55°C, 30 s; 72°C, 60 s) with the following oligonucleotide primer sets: IL-6 sense: 5′-TGGAGTCACAGAAGGAGTGGCTAAG-3′, IL-6 antisense: 5′-TCTGACCACAGTGAGGAATGTCCAC-3′, β-actin sense: 5′-TGGAATCCTGTGGCATCCATGAAAC-3′, β-actin antisense: 5′-TAAAACGCAGCTCAGTAACAGTAACAGTCCG-3′. The amplified DNA fragments were as expected 154 bp for IL-6 and 359 bp for β-actin. Ten μl of final product were loaded onto a composite gel containing 1% Nusieve/1% agarose (FMC Co. Rockland, ME, USA), electrophoresed, and visualized by ethidium bromide staining.

2.5. Nuclear extract preparation and electrophoretic mobility gel shift assays (EMSAs) for NF-κB

Nuclear extract preparation and DNA binding conditions for the EMSAs were as previously described [12]. Briefly cells were homogenized in buffer A (0.5 M sucrose, 10 mM Tris-HCl, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA) following exposure to ionizing radiation at a dose of 10 Gy ,and nuclei were collected by centrifugation at 2,000 rpm for 10 min. The pellet was homogenized in buffer B (5% glycerol, 10 mM Tris-HCl (pH 7.9), 400 mM NaCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA) and incubated for 30 min. The debris was removed by centrifugation at 14,000 rpm for 45 min, and the supernatant was taken as the nuclear fraction. Following dialysis for 12 h, the extract (nuclear protein) was centrifuged at 8,000 rpm for 10 min. Five mg of nuclear proteins were incubated with double-stranded 35 bps ^{32}P-radiolabeled oligodeoxynucleotides encoding the Ig κ chain,the NF-κB concensus sequence (5×10^4 cpm; approximately 1.0 ng) in buffer containing 10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 1.0 mM EDTA, 5% glycerol, 1.0 mM DTT and 2.0 μg poly (dI-dC) (Sigma Chemical Co. St. Louis, MO, USA) in a final volume of 20 μl. Mixtures were incubated at 37°C for 30 min and then analyzed on native 6% polyacrylamide gels using Tris-boric acid/EDTA buffer. Gels were dried and exposed for auto-

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radiography. For the competetion assays, a 40-fold molar excess of unlabeled probe was added as a competetor 30 min prior to addition of the labeled probe.

2.6. Neutralization of IL-6

To neutralize IL-6 in the supernatant of the cells, Ten μg/ml gout anti-murine IL-6 antibody (R & D Systems, Inc., Minneapolis, MN) was used as previously described [13]. Ten μg/ml of gout IgG were used as a negative control.

3. Results and discussion

3.1. Induction of neurite outgrowth by ionizing radiation

Neurite outgrowth was detectable at 5 Gy and reached a maximum at 10–20 Gy 24 h following exposure to ionizing radiation in the presence of 10% serum (Fig. 1A). The maximal effect of serum on neurite outgrowth occured at a concentration of 10–20%. Neurite outgrowth was not detectable at a concentration of 0–1% (Fig. 1B). This suggests that the induction of neurite outgrowth in PC12 cells by ionizing radiation requires serum.

3.2. Expression of IL-6 mRNA by ionizing radiation

It had been already reported that IL-6 can induce neurite outgrowth in PC12 cells [2]. We examined the expression of IL-6 mRNA in PC12 cells using RT-PCR with the irradiation. Although no expression of IL-6 mRNA was seen in PC12 cells exposed to 0 Gy with or without 10% serum or 5 Gy without serum (Fig. 2, lanes 1, 2, 3), marked expression of IL-6 mRNA was observed with 5 Gy and 15 Gy in the presence of 10% serum (Fig. 2, lanes 4,6). PC12 cells treated with 15 Gy in the absence of 10% serum expressed small amounts of IL-6 mRNA (Fig. 2, lane 5). This suggests that the expression of IL-6 mRNA in PC12 cells by ionizing radiation also requires serum.

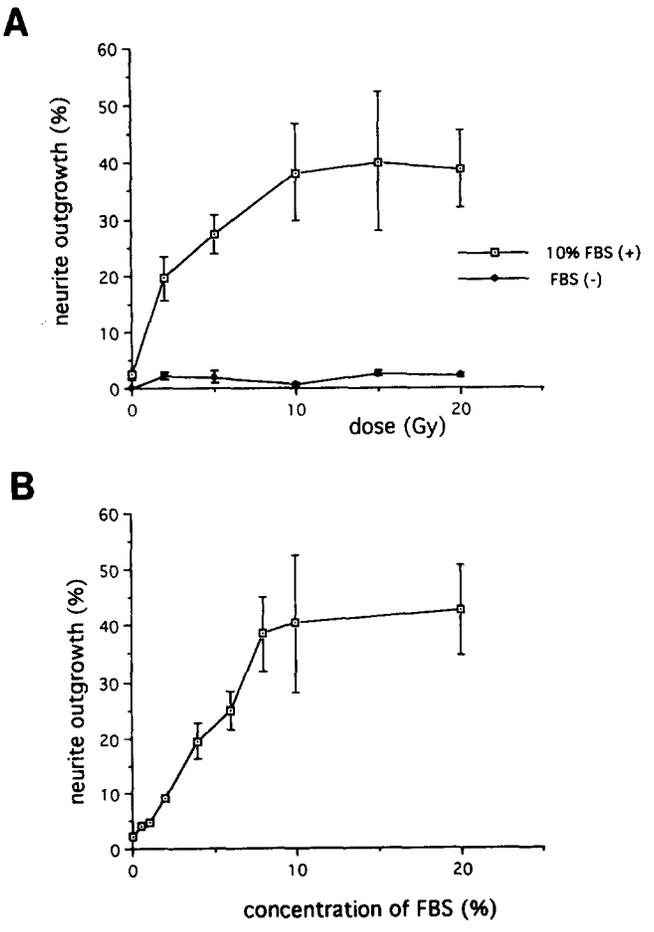

Fig. 1. (A) Dose dependent effect of ionizing radiation on differentiation of PC12 cells. Cells exposed to various dose of X-rays were cultured for 24 h in the absence of FBS or the presence of 10% FBS. (B) Effect of concentration on the differentiation of PC12 cells. Cells exposed to X-ray at a dose of 15 Gy were cultured for 24 h. The extent of neurite outgrowth was then determined.

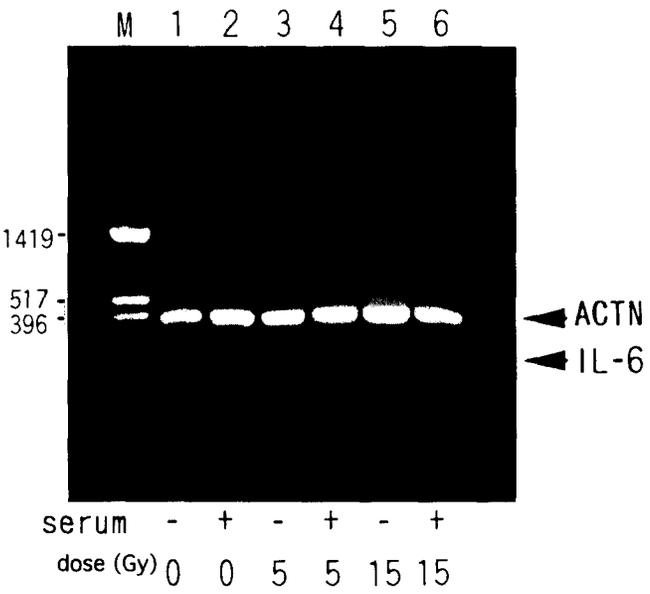

Fig. 2. Cells exposed to X-ray at doses of 0 Gy (lanes 1, 2),5 Gy (lanes 3, 4) and 15 Gy (lanes 5, 6) were cultured for 15 h in the absence of FBS (lanes 1, 3, 5) or in the presence of 10% FBS (lanes 2, 4, 6). Expression of IL-6 mRNA was determined by RT-PCR as described in section 2.

3.3. Induction of NF-κB activation by ionizing radiation

To study the effect of ionizing radiation on the activity of NF-κB, EMSAs with nuclear proteins isolated from PC12 cells were performed. Incubation of the ^{32}P-radiolabeled probe con-

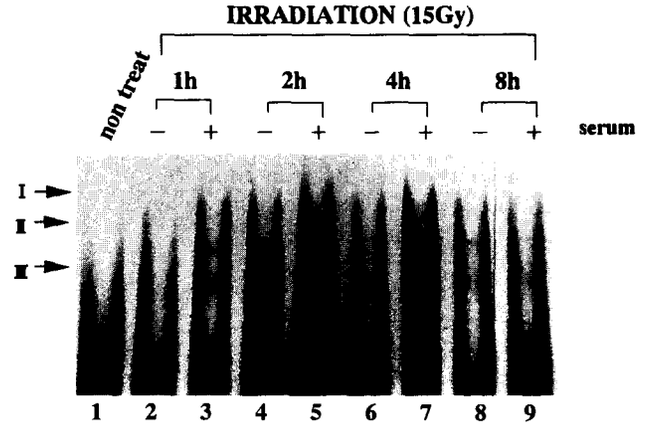

Fig. 3. EMSAs were performed with nuclear proteins (5 μg) isolated from PC12 cells exposed to X-rays. Cells exposed to X-rays at a dose of 15 Gy were cultured for 1 h (lanes 2, 3), 2 h (lanes 4, 5), 4 h (lanes 6, 7) and 8 h (lanes 8, 9) in the absence of FBS (lanes 2, 4, 6, 8) or in the presence of 10% FBS (lanes 3, 5, 7, 9).

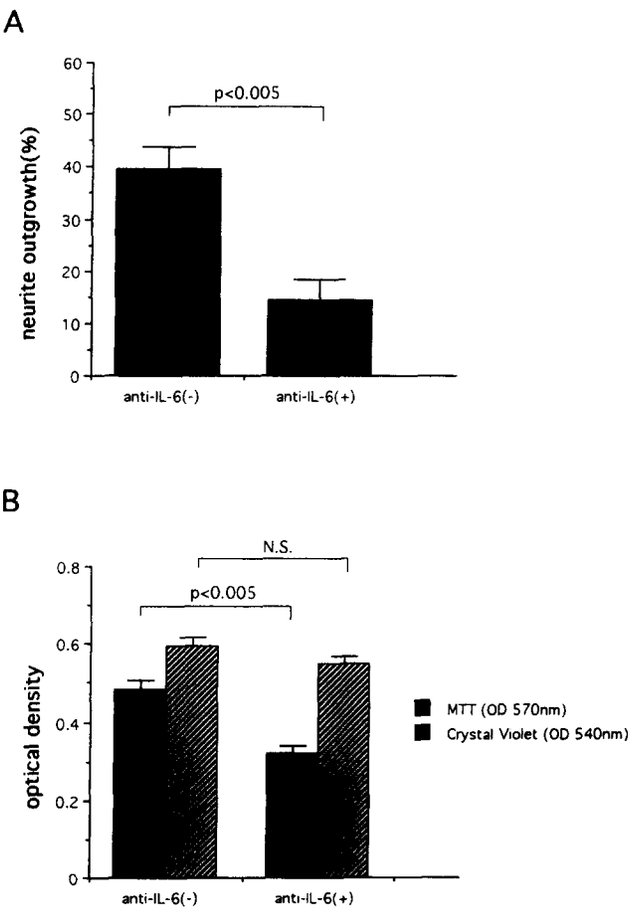

Fig. 4. (A) Inhibition of radiation-induced neurite outgrowth of PC12 cells by the addition of anti-murine IL-6 antibody. PC12 cells X-irradiated at a dose of 15 Gy were cultured for 24 h in DMEM supplemented with 10% FBS in the presence of 10 μg/ml anti-IL-6 antibody (anti IL-6 (+)) or in the absence of anti-IL-6 antibody (anti IL-6 (−)). The extent of neurite outgrowth was then determined. (B) Inhibition of MTT reduction in X-irradiated PC12 cells by anti-IL-6 antibody. Cells were treated as in Fig. 4A. MTT reduction assay for determination of redox activity and Crystal violet staining for determination of viablity were performed. Cell redox activity and viability were quantified as the absorbance at 570 nm for MTT and 540 nm for Crystal violet.

taining the NF-κB binding site with the nuclear proteins retarded the electrophoretic migration of three distinct bands (Fig. 3: arrows I, II, III). Complete inhibition of the three bands was obtained with a 40-fold excess of unlabeled probe (data not shown). Induction of NF-κB binding to DNA in irradiated PC12 cells was increased more in medium containing 10% FBS than in serum free medium (Fig. 3).

3.4. Inhibition of neurite outgrowth and decrease of MTT reduction by neutralization of IL-6

As our findings suggested a close relationship between IL-6 production by PC12 cells with exposure to ionizing radiation and neurite outgrowth, we examined the effect of neutralization of IL-6 on the induction of neurite outgrowth, cellular viability and redox activity. Ten mg/ml anti-murine IL-6 antibody inhibited neurite outgrowth (Fig. 4A). Although the neutralization of IL-6 did not affect the viability of PC12 cells, decreased MTT redox activity was seen (Fig. 4B) 24 h following exposure to ionizing radiation in the presence of 10% FBS.

Our findings demonstrate that irradiated PC12 cells incubated in medium containing serum can undergo neurite outgrowth, with the induction of neurofilaments as a differentiation marker (data not shown). This effect was inhibited by the neutralization of IL-6 in the supernatant. Recent studies have demonstrated that treatment of mammalian cells with ionizing radiation is associated with gene expression. Ionizing radiation stimulates the binding of preexisting NF-κB to DNA and increases the expression of the NF-κB gene [6]. It has been reported that ionizing irradiation transcriptionally activates the IL-6 gene, and that this transcriptional activation involves the activation of NF-κB [5]. It also has been reported that IL-6 transcription is induced by serum [14,15]. Our data suggest that ionizing radiation and serum synergistically stimulate NF-κB activation and IL-6 transcription. The production of IL-6 appears then to induce the neuronal differentiation of these cells.

Ionizing radiation can result both in neoplastic transformation and lethal events in mammalian cells, thought to occur through the formation of DNA-damaging free radicals. The neutralization of IL-6 decreased MTT reduction in irradiated PC12 cells. Rescue of redox activity by IL-6-induced neuronal differentiation may prevent cells from radiation-(free radical)-induced lethal damage.

References

[1] Green, L.A. and Tischler, A.S. (1982) Adv. Cell. Neurobiol. 3, 373–414.
[2] Satoh, T., Nakamura, S., Taga, T., Matsuda, T., Hirano, T. Kishimoto, T. and Kaziro, Y. (1988) Mol. Cell. Biol. 8, 3546–3549.
[3] Hallahan, D.E., Spriggs, D.R., Beckett, M.A., Kufe D.W. and Weichselbaum, R.R. (1989) Proc. Natl. Acad. Sci. USA. 86, 10104–10107.
[4] Witte, L., Fuks, Z., Haimonovitz-Freidman, A., Vlodavski, I., Goodman,D.S. and Eldor, A. (1989) Cancer Res. 49, 5066–5072.
[5] Brach, M.A., Grub, H.J, Kaisho, T., Asano, Y., Hirano, T. and Herrmann, F. (1993) J. Biol. Chem. 268, 8466–8472.
[6] Brach, M.A., Hass, R., Sherman, M.L., Gunji, H., Weichselbaum, R. and Kufe, D. (1991) J. Clin. Invest. 88, 691–695.
[7] Sen, R. and Baltimore, D. (1986) Cell 46, 705–716.
[8] Hansen, M.B., Nielson, S.E. and Berg, K. (1989) J. Immunol. Methods 119, 203–210.
[9] Tsujimoto, M., Yip, Y.K. and Vilcek, J. (1985) Proc. Natl. Acad. Sci. USA, 82, 7626–7630.
[10] Kitajima, I., Yamamoto, K., Sato, Y., Nakajima, T., Maruyama, I., Osame, M. and Nishioka, K. (1991) J. Clin. Invest. 88, 1315–1322.
[11] Chomczynski, P. and Sacch, N. (1987) Anal. Biochem. 162, 156–159.
[12] Kitajima, I., Shinohara, T., Minor, T., Bilakovics, J., Brown, D.A., Xu, X. and Nerenberg, M. (1992) Science 258, 1792–1794.
[13] Maeda, Y., Matsumoto, M., Hori, O., Kuwabara, K., Ogawa, S., Yan, S.D., Ohtsuki, T., Kinoshita, T., Kamada, T. and Stern, D.M. (1994) J. Exp. Med. 180, 2297–2308.
[14] Ray, A., Tatter, S.B., May, L.T. and Sehgal, P.B. (1988) Proc. Natl. Acad. Sci. USA 85, 6701–6705.
[15] Ray, A., Sassone-Corsi, P. and Sehgal, P.B. (1990) Mol. Cell. Biol. 9, 5537–5547.