Letter

Xray Irradiation Promotes Nerve Growth Factor-induced Neurite Extension in PC12 Cells

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Received October 30, 2015
Accepted January 8, 2016

Nerve growth factor (NGF) induces neurite extension in PC12 cells, a well-known model of neuronal differentiation. Previous reports have shown that irradiation activates the extracellular signal-regulated kinase 1/2 (ERK), which is a mainstream pathway for NGF signaling. This suggests that the neurite extension may possibly be enhanced by Xray irradiation. Therefore, we observed NGF-induced neurite extension with Xray irradiation in PC12 cells. At the start of the NGF treatment, Xray irradiation was performed simultaneously for the first 5 min. After 5 days, the irradiation at 500 mGy promoted NGF-induced neurite extension, and increased phosphorylation of ERK, but not of the NGF receptor. The phosphorylation of epidermal growth factor (EGF) receptor, a stimulatory growth signal in PC12 cells, was also enhanced by irradiation. Interestingly, AG1478, an inhibitor of EGF receptor tyrosine kinase, inhibited the irradiation-induced promotion of neurite extension. These results suggest that irradiation activate EGF receptor tyrosine kinase and promote NGF-induced neurite extension via the cross talk between NGF and EGF signaling.

Key Words: neural differentiation, nerve growth factor, neurite extension, Xray irradiation, PC12 cells

1. Introduction

Radiation therapy is effective in the treatment of malignant brain tumors, which are not easily treated surgically. Although radiation suppresses tumor cell proliferation and is an effective treatment, it is difficult to completely avoid irradiation of normal nerve tissues that are the passing point of radiation to the lesion site. As a result, side effects caused by radiation therapy such as cognitive impairment and brain atrophy can occur.1) In addition, the normal central neurons around the radiation field, may be exposed to considerably lower doses of radiation (scattered radiation) than that used for the treatment. However, the effects of low-dose radiation on neurons have not been elucidated well.

The binding of the major nerve differentiation factor, the nerve growth factor (NGF), to the receptor tyrosine kinases causes phosphorylation of the intracellular domain of its receptor.2) Following phosphorylation, Ras, mitogen-activated protein (MAP) kinase cascade, and extracellular signal-regulated

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kinase 1/2 (ERK) are activated, which induce the differentiation of neural cells. Several studies have reported that considerably lower-dose radiation activates intracellular ERK, although not through the receptor activation by ligands mentioned above. Taken together, the promotion of NGF-effect by simultaneous irradiation with NGF-treatment can be hypothesized. If this hypothesis is correct, a lower-dose radiation treatment may be useful for promoting the regeneration of damaged nerve tissues. In fact, there are some studies showing that low-dose irradiation exerts accelerating effects on the functional recovery of damaged spinal nerves.

PC12 cells, derived from a pheochromocytoma of the rat adrenal medulla, differentiate into neuron-like cells when ERK is continuously activated by NGF stimulation. Therefore, NGF-induced neurite extension in PC12 cells has been widely used as a differentiation model of neurons. In this study, we examined whether NGF-induced neurite extension in PC12 cells is enhanced by irradiation with an aim to verify the above mentioned hypothesis.

2. Materials and methods

2-1 Reagents

Reagents used were as follows: NGF 2.5S (Alomone Labs), anti-phosphorylated ERK1/ERK2 rabbit-poly antibody (R & D systems), anti-phosphorylated Trk A rabbit-poly antibody (Trk A is a high affinity nerve growth factor receptor, Santa Cruz Biotechnology), AG1478 (a specific inhibitor of the EGF-receptor tyrosine kinase, Cell Signaling Technology).

2-2 Neuronal extension assay

Rat pheochromocytoma cells (PC12) were maintained as previously described. The cells were cultured in a 75 cm² flask at 37°C in a mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium (DF medium 1 : 1, vol/vol) containing 15 mM HEPES buffer, pH 7.4, 50 units/mL penicillin G, 0.1 mg/mL streptomycin sulfate, 5% horse serum, and 5% fetal calf serum in 5% CO₂ humidified atmosphere.

For neuronal extension assay, the cells were cultured in the serum-containing medium in 35-mm collagen-coated dishes for 3h. Treatment of the cells with 50 ng/mL NGF was performed under serum-free medium (DF medium containing 15 mM HEPES buffer, pH 7.4, 50 units/mL penicillin G, 0.1 mg/mL streptomycin sulfate and ITS Premix [Becton, Dickinson]), for 5 days. Lengths or numbers of neurites were quantified by nerve axons image analysis software (Kurabo, Osaka, Japan), and were expressed as a relative ratio to the non-irradiated group.

2-3 Xray irradiation

The Xray irradiations by the irradiation system (Faxitron CP-160) were performed for the first 5 min simultaneously with NGF stimulus onset. The radiation dose-rate was 20 mGy/min (total dose 100 mGy) or 100 mGy/min (total dose 500 mGy).

2-4 Western blot analysis

Protein extractions from the cells were performed using the CytoBuster™ Protein Extraction Reagent (Merck Millipore) and were concentrated or diafiltered with Amicon Ultra devices (Merck Millipore). Cell lysates were subjected to SDS-PAGE (12.5%), followed by western blotting. The target proteins were visualized using a WesternBreeze® Chemiluminescent Kit (Thermo Fisher Scientific).

3. Results and discussion

Under NGF stimulation, PC12 cells extended their neurites and clearly differentiated into neuron-like cells 5 days later (Fig. 1a). With the Xray irradiation for 5 min immediately after the NGF addition, the effect of NGF on neurite extension was increased in a
dose-dependent manner (Fig. 1a). The quantification of neurite extension showed approximately 1.4-fold and 2.5-fold increase in the length and number of neurites, respectively, by irradiation of 500 mGy Xrays compared to those in the non-irradiated group (Fig. 1b, c). By contrast, the Xray irradiation did not induce neurite extension of the cells that were not stimulated by NGF (Fig. 1a–c). Protein expression analysis showed increased expression of microtubule-associated protein 2 (MAP2), a major protein constituting the neurites (Fig. 2a) which supports the above morphological change of enhanced neurite extension by irradiation. These results suggest that
X-ray irradiation enhances NGF-induced neurite extension in PC12 cells.

Next, in order to analyze the effect of irradiation on the activation of signals in the NGF signaling pathway, levels of phosphorylated NGF receptor were analyzed. In the western blotting analysis, the phosphorylation levels of NGF receptor with NGF stimulation did not change by X-ray irradiation (Fig. 2b). By contrast, the levels of phosphorylated ERK, a downstream signaling protein from the NGF receptors, were further elevated by irradiation during NGF stimulation (Fig. 2c). These results suggest that the enhanced NGF-induced neurite extension by irradiation is not caused by the activation of NGF receptor signals, but by the activation of downstream signals.

EGF activates its receptors in PC12 cells to cause phosphorylation of ERK, which is involved in the downstream pathway of EGF receptors, similar to the activation of NGF receptors. Low-dose radiation has been reported to induce phosphorylation of EGF receptors. These facts suggest that the enhancement of NGF-induced neurite extension by irradiation may be exhibited by the additive effect of the activation of ERK via activation of EGF receptors by irradiation. In order to verify this possibility, we examined the phosphorylation levels of EGF receptor. Western blot analysis showed elevated phosphorylation of EGF receptors by irradiation.
In addition, when this phosphorylation was suppressed by an EGF receptor tyrosine kinase inhibitor, AG1478, the enhancement of NGF-induced neurite extension by irradiation was also suppressed (Fig. 3a–c). By contrast, AG1478 did not affect the phosphorylation levels of the NGF receptor (Fig. 3a) and the NGF-induced neurite extension without the irradiation (Fig. 3b, c). These results suggest that the enhancement of NGF-induced neurite extension by X-ray irradiation is caused through the activation of EGF receptors.

NGF, which causes PC12 cells to differentiate into neuron-like cells, is an inducer of cell differentiation, whereas EGF, which promotes proliferation of PC12 cells, is a cell growth factor. Despite different directions of cell fate induced by these factors, both include ERK activation in their downstream signaling pathways. The cell fate directed by each factor is considered to be determined by a difference in the activation period of ERK. NGF stimulation causes persistent ERK activation for a long period, leading to differentiation, while EGF stimulation leads to proliferation by transient ERK activation. In this study, irradiation did not directly cause the activation of NGF receptors. Furthermore, although the EGF receptor activation and ERK activation were induced by irradiation alone, without NGF stimulation, neurite extension was not induced by irradiation alone. However, the EGF receptor activation by irradiation increased induction of NGF-induced differentiation. Therefore, our results suggest that the irradiation effects observed in this study were expressed not through the increase in the first signal by NGF, but through an additive increase in NGF-induced ERK activation via EGF receptors (Fig. 4). Consequently, the continuous ERK activation was increased, which

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**Fig. 4** Hypothesis of the promotion mechanism of the NGF-induced neurite extension by irradiation in PC12 cells. The activation of the EGF receptor by irradiation starts from the flow along a ligand-independent pathway and exerts an additional effect on the persistent activation of Erk by NGF, resulting in the promotion of the neurite extension.
resulted in the enhancement of NGF-induced neurite extension. The effects observed in this study may be considered the same as increasing the amount of NGF.

However, according to this hypothesis, the cell proliferation pathway is also activated through activation of EGF receptor by irradiation; thus, why only the enhanced differentiation through activation of NGF receptor was observed cannot be explained. It has been reported that the activation of EGF receptor by irradiation promotes maintenance of cell survival via stimulation of signals in the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway.\(^{14}\) By contrast, there is no report demonstrating that low-dose radiation promotes cell growth activity of EGF. Taken together, in the case of activation by ligands of EGF receptor and by irradiation, the post activation signals may follow different pathways. In fact, a study has shown that the ligand-independent activation of EGF receptor, such as activation by irradiation, is involved in DNA repair caused by stimulation inducing genotoxicity, rather than in cell growth.\(^{15}\) However, many points regarding the pathway after EGF receptor activation are unknown, such as the mechanism by which the ligand-dependent activation is distinguished from the ligand–independent activation or the mechanism by which the ligand-independent pathway exerts an additive effect on the NGF pathway (Fig. 4). Further detailed studies are necessary regarding this.

In this study, we first found that low-dose radiation enhances the differentiation of PC12 cells into neuron-like cells by NGF. Due to low sensitivity of neurons to radiation, there are only a few studies on the effects of radiation on neurons. Therefore, the results of this study provide significant information for analyzing the effects of low-dose radiation on central nerve cells. However, the effects of other EGF receptor tyrosine kinase inhibitors such as gefitinib should be examined, to confirm the hypothesis of this study.

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和文要旨

X線照射はPC12細胞における神経成長因子誘導の神経軸索伸長を促進する
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X線500mGyの照射により，神経成長因子（NGF）によるPC12細胞の神経軸索伸長が促進された。このとき，NGFの主要な細胞内シグナル分子ERKのリン酸化は増加していたが，NGF受容体自身のリン酸化は亢進していなかった。興味深いことに，増殖シグナルを活性化する上皮成長因子（EGF）の受容体のリン酸化が照射によって亢進しており，これを阻害剤にて抑制したところ，照射によるNGF誘導神経軸索伸長の促進は抑えられた。以上の結果から，X線照射によるEGF受容体のリン酸化の亢進がNGFシグナルとのクロストーク経路を介して，NGF誘導の神経軸索伸長を促進していることが示唆された。

（2015年10月30日 受付）
（2016年 1月 8日 受理）