J. Radiat. Res., 52, 369–373 (2011)

Short Communication

Norepinephrine Enhances Radiosensitivity in Rat Ileal Epithelial Cells

Mutsumi MATSUU-MATSUYAMA[1*], Kumio OKAICHI[2], Kazuko SHICHIJO[1], Toshiyuki NAKAYAMA[4], Masahiro NAKASHIMA[1,3] and Ichiro SEKINE[5]

Norepinephrine (NE)/Radiosensitivity/Rat ileal epithelial cells (IEC-18).

We previously reported that the apoptosis index in jejunal crypt cells after X irradiation was greater in spontaneously hypertensive rats than in Wistar-Kyoto rats. Moreover, these same cells showed a suppression of apoptosis when reserpine was administered to induce sympathetic dysfunction in spontaneously hypertensive rats or Wistar-Kyoto rats.[1)] Whether the hyperfunction of the sympathetic nervous system is involved in the high susceptibility of the jejunal crypt cells to radiation-induced apoptosis was the subject of this study. The effect of norepinephrine (NE) on cell survival was examined using the colony formation assay after X-ray irradiation of rat ileal epithelial cells (IEC-18). The addition of 1 μM NE decreased the surviving fraction of cells irradiated with 6 Gy from 37% to 8%. The radiosensitivity of IEC-18 cells was enhanced by the addition of 1 μM of NE. The irradiation and treatment with NE also resulted in an increased cellular apoptotic rate. These results showing enhanced radiosensitivity of rat ileal epithelial cells by NE suggest that NE may be one of the factors which aggravate acute radiation injury in the intestine.

INTRODUCTION

Spontaneously hypertensive rats, originally derived from Wistar Kyoto rats, are a well-known model for studying essential hypertension in humans. Untreated spontaneously hypertensive rats show sympathetic facilitation in various organs compared to Wistar-Kyoto rats. We previously reported that the apoptosis index in jejunal crypt cells after X irradiation was greater in spontaneously hypertensive rats than in Wistar-Kyoto rats. Further, the administration of reserpine to induce sympathetic dysfunction of spontaneously hypertensive rats or Wistar-Kyoto rats resulted in suppression of apoptosis.[1)] We also reported that radiation evoked a stronger response to NE in the jejunum and colon of spontaneously hypertensive rats than in Wistar-Kyoto rats.[2)] The survival rate after irradiation of spontaneously hypertensive rats was lower than for Wistar-Kyoto rats, and weight loss, appetite loss and morphological changes in the jejunum were greater in spontaneously hypertensive rats than in Wistar-Kyoto rats.[2)] These findings suggested that spontaneously hypertensive rats have a greater susceptibility to radiation-induced apoptosis in the jejunum than Wistar-Kyoto rats. X irradiation caused greater activities in autonomic nervous function and severe radiation injury in spontaneously hypertensive rats. Thus, sympathetic hyperfunction may be associated with a high susceptibility to radiation, including radiation injury, radiation sickness and radiation-induced apoptosis of the jejunal crypt cells.

The sympathetic nervous system in the intestine modulates motility,[3)] mucosal blood flow, secretion[4)] and immune function.[5)] NE is the primary transmitter of the sympathetic nervous system. In regard to NE and apoptosis, exposure to catecholamines induces apoptosis in the thymus, spleen and lymph nodes, and acts via the alpha-adrenoreceptor.[6)] It was also reported that NE, acting via the beta-adrenergic pathway, stimulates apoptosis in adult rat cardiac myocytes in vitro, and NE-stimulated apoptosis of cardiac myocytes might contribute to the progression of myocardial failure.[7)] Another group suggested that NE induces apoptosis in neonatal rat endothelial cells via down-regulation of Bcl-2 and activation of beta-adrenergic and caspase-2 pathways.[8)]

Other than our studies, there are only a few reports exam-

*Corresponding author: Phone: +81-95-819-7107,
Fax: +81-95-819-7108,
E-mail: mutsumi@nagasaki-u.ac.jp
[1]Department of Tumor and Diagnostic Pathology; [2]Department of Radiation Biophysics; [3]Tissue and Histopathology Section, Atomic Bomb Disease Institute; [4]Department of Pathology, Nagasaki University Graduate School of Biomedical Sciences 1-12-4 Sakamoto, Nagasaki 852-8523, Japan; [5]Nagasaki Red Cross Blood Center, Nagasaki, Japan.
doi:10.1269/jrr.10155

ining the relationship between the radiosensitivity in intestinal epithelial cells and NE.[1,2] The intestinal tract shows a high sensitivity to ionizing radiation due to rapid cell turnover, and exposure to ionizing radiation results in the rapid death of crypt cells by apoptosis. Damage to the crypts is accompanied by functional changes, including malabsorption, which are expressed clinically as acute bowel reactions. The nontransformed rat ileal epithelial cells (IEC-18), derived from crypts of the intestine,[9] have provided an in vitro model to examine intestinal epithelial cell migration, differentiation and proliferation.[10] To test the sensitivity of the sympathetic nervous system to radiation, we examined the effect of NE on cell survival after X-ray irradiation of IEC-18 cells using the colony formation assay.

MATERIALS AND METHODS

Cell culture

IEC-18 cells, obtained from American Type Culture Collection (Rockville, MD, USA), were grown in high-glucose (4.5 g/L) Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 5% fetal bovine serum (FBS) (Equitech-Bio, Kerriville, TX, USA) and 0.1 units / ml insulin (Sigma, St.Louis, MO, USA) at 37 degrees in a water-saturated atmosphere of 95% air and 10% CO_2. IEC-18 cells used in this study were before passage 15 in our laboratory.

Irradiation

X irradiations were performed with a Toshiba EXS-300 X-ray, 200 kV, 15 mA apparatus with 0.5 mm aluminum filter at a dose rate of 0.694 Gy/min for the study of survival curves for IEC-18 cells treated with different doses of NE and with or without 6 Gy irradiation (Fig. 1B), and for the study of TUNEL staining (Fig. 2), or a Toshiba ISOVOLT TITAN 32 X-ray, 200 kV, 15 mA apparatus with 0.5 mmAl + 5 mmAl filter at a dose-rate of 0.8903 Gy/min for the study of survival curves for IEC-18 cells treated with different doses of radiation with or without 1 μM NE (Fig. 1C). X-ray quality (spectrum) of Toshiba ISOVOLT TITAN 32 X-ray was adjusted to that of Toshiba EXS-300 X-ray by adding 5 mmAl filter. The effective energy of ISOVOLT TITAN 32 was considered higher than that of EXS-300 because the system of tube voltage formation was different.

Colony formation assay

Semi-confluent IEC-18 cells were incubated in DMEM medium with 0.1% FBS medium for 24 h in 100 mm dishes. Cells were then trypsinized and the cells seeded were adjusted to yield approximately 100 colonies per dish after treatment. The cell suspension was counted using a Nucleo Counter (Chemometec A/S, Denmark). NE was obtained from Sigma, and was dissolved in PBS.

IEC-18 cells were treated with NE for 2 h before irradiation and then exposed to X-ray. After irradiation, the cells were incubated for 2 days with 0.1% FBS medium and then 5% FBS was added for 12 days. The cells were then fixed with neutral-buffered formalin and stained with Giemsa for counting of cell colonies.

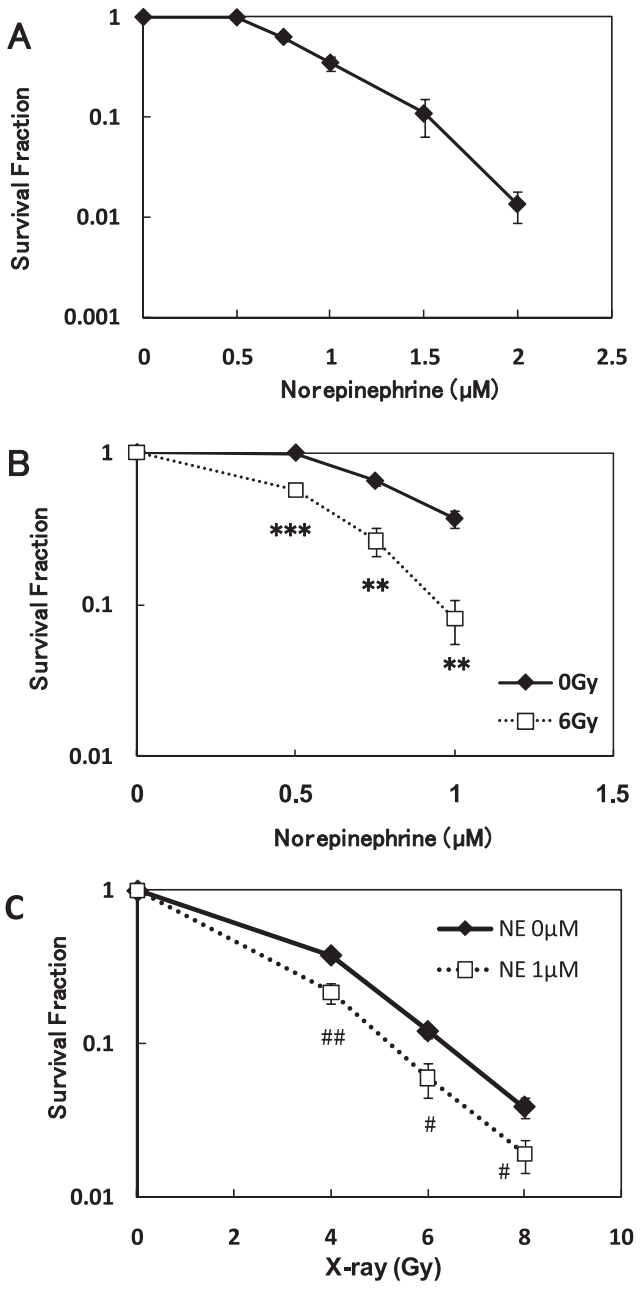

Fig. 1. (A) Survival of IEC-18 cells incubated with different concentrations of NE. (B) Surviving fraction after treatment with different concentrations of NE after 6 Gy irradiation. (C) Survival curves for IEC-18 cells treated with different doses of radiation with or without 1 μM NE. Data shown are means ± SE from three to five independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ relative to 0 Gy. $\#p < 0.05$, $\#\#p < 0.01$ relative to NE 0 μM.

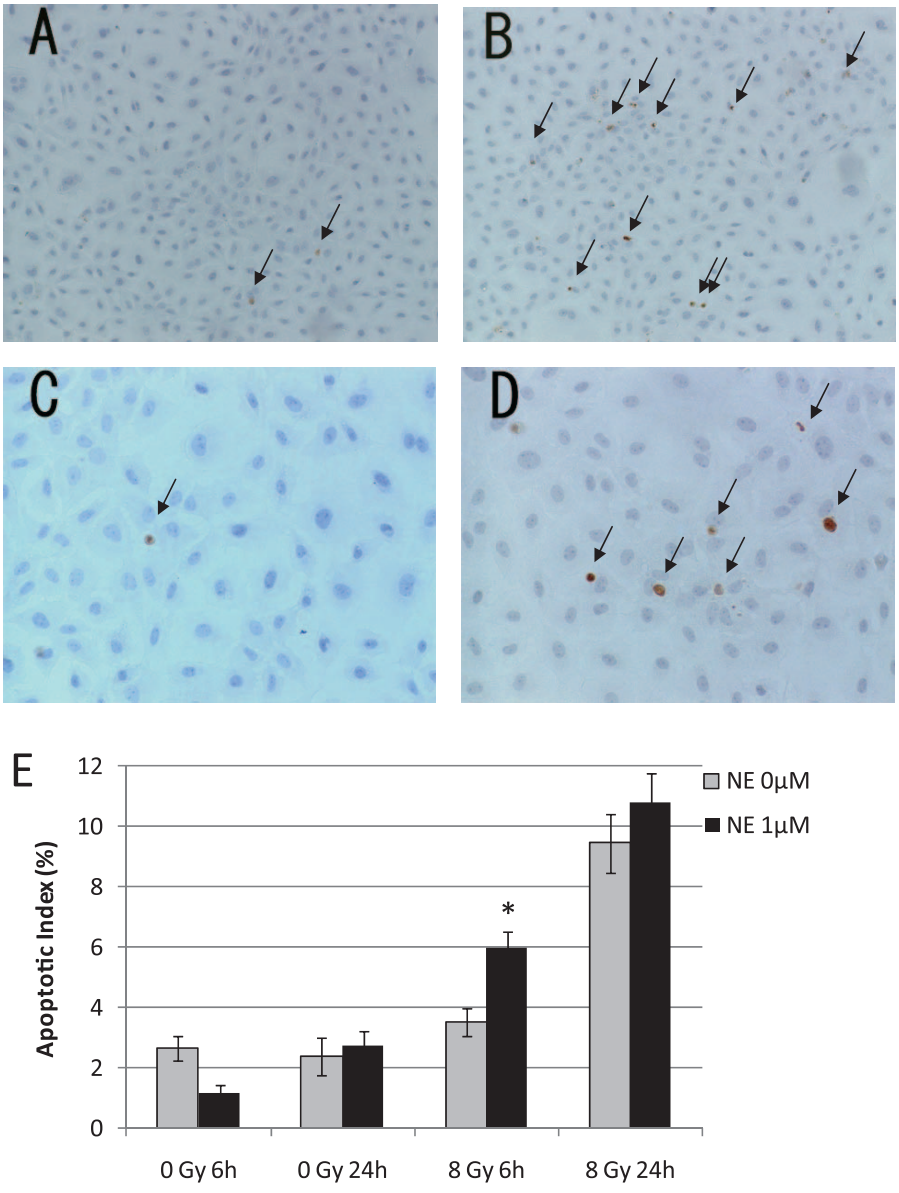

Fig. 2. Apoptosis of IEC-18 cells was detected by TUNEL staining which was performed 6 h after 8 Gy X irradiation. Cells in (A) and (C) were untreated while cells in (B) and (D) were treated with 1 μM NE. Cells in (A) and (B) were taken under low magnification (×100), while (C) and (D) were taken under high magnification (×400). Apoptotic cells are indicated by the arrows. (E) Apoptotic rates at 6 h and 24 h following irradiation with 8 Gy of cells untreated or treated with 1 μM NE. Data represent the mean ± SEM of five to nine separate fields. * $p < 0.05$ relative to NE 0 μM.

TUNEL staining

Semi-confluent IEC-18 cells were incubated in 60 mm dishes with 0.1% FBS medium for 24 h and treated with or without 1 μM NE for 2 h before irradiation. Six and 24 hours after irradiation, apoptosis was detected using ApopTag Peroxidase *In Situ* Apoptosis Detection Kit (Chemicon International Inc., Temecula, CA, USA). The cells were fixed in 1% paraformaldehyde (Wako Pure Chemical Industries, Ltd., Osaka, Japan), in PBS, pH 7.4 for 10 minutes at room temperature. Endogenous peroxidase was blocked in 3% hydrogen peroxide. The cells were incubated with terminal deoxynucleotidyl transferase (TdT) enzyme for 1 hour at 37 degrees, and with anti-digoxigenin conjugate for 30 minutes at room temperature. The reaction sites for the TUNEL assay were visualized using DAB (Dako, Tokyo, Japan). Cell nuclei were stained with 0.5% methyl green, and apoptotic and total cells were then counted under ×400 magnification. Five to nine representative microscopic fields

were analyzed. For 6 and 24 h after irradiation, at least 200 and 100 cells respectively, were counted in each field. The results were expressed as a percentage of the apoptotic cells.

Statistical analysis

All data are expressed as mean ± standard error (SE) obtained from three independent experiments with similar patterns. The mean values of two groups were assessed by Student's *t*-test, and *p* values less than 0.05 were considered significant.

RESULTS AND DISCUSSION

NE treatment of IEC-18 cells resulted in the decrease of cell survival in a dose dependent manner (Fig. 1A). Cell survival decreased slightly when cells were incubated with 1 μM NE but thereafter a rapid decrease followed as the dose of NE was increased to 2 μM.

Figure 1B shows survival curves for cells that received radiation after treatment with NE. The survival fraction of cells after 6 Gy irradiation was based on 0 μM NE, which was 0.293 ± 0.072 relative to the survival fraction of cells with 0 Gy. Cell survival fraction of cells treated with 0.5, 0.75 and 1 μM NE decreased with 6 Gy irradiation ($p < 0.001$ at 0.5 μM, $p < 0.01$ at 0.75 and 1 μM NE relative to 0 Gy). Figure 1C shows that the addition of 1 μM NE decreased the clonogenic cell survival at 4 Gy ($p < 0.01$), 6 Gy ($p < 0.05$) and 8 Gy ($p < 0.05$) compared to 0 μM NE . The survival fraction of cells with 1 μM NE was based on 0 Gy, which was 0.83 ± 0.067 relative to the survival fraction of cells with 0 μM NE 0 Gy.

NE concentration of 1 μM increased the apoptotic rate from 3.5% to 6.0% at 6 h after 8 Gy irradiation ($p < 0.05$) as detected by TUNEL staining (Fig. 2). The apoptotic rate of 0 μM of NE was increased to 9.5% at 24 h after irradiation, but there was no difference with 1 μM of NE (10.8%) (Fig. 2E). There was no significant difference in the background (0 Gy) levels of the apoptotic rate between 0 μM and 1 μM of NE (Fig. 2E).

In the previous study, sympathetic hyperfunction was considered to be highly susceptible to radiation,[1,2] and the TUNEL assay was performed to evaluate the rate of apoptosis in radiation induced cell death as well as by trypan blue staining.[11] In this study, the effect of norepinephrine on survival of IEC-18 cells after X-ray irradiation was examined. We have shown that norepinephrine decreased cell survival of IEC-18 cells after irradiation (Fig. 1). To detect DNA fragmentation by apoptosis, we performed the TUNEL assay and showed that norepinephrine enhanced the radiation-induced apoptosis of IEC-18 cells at 6 h after irradiation (Fig. 2). The decrease of cell survival is in part due to apoptosis, but also may due to senescence or necrotic death in fewer colonies. The decrease of cell survival in norepinephrine treated cells after irradiation may be in part attributed to the increase of radiation-induced apoptosis by norepinephrine.

The activation of the sympathetic nervous system leads to the release of norepinephrine from nerve endings acting on the heart, blood vessels, respiratory centers, and other sites. The ensuing physiological changes constitute a major part of the acute stress response, characterized by an almost instantaneous surge in heart rate, blood pressure, sweating, breathing, and metabolism, and a tensing of muscles. Noerpinephrine is an agonist of α-, β- adrenoceptors. Alpha adrenoceptors have been characterized into two groups designated α_1, α_2. Alpha adrenoceptors have been one of the most widely studied families of receptors because of their importance in control of blood pressure and blood flow, neural modulation, digestion, micturition, airways, reproduction, pupil diameter, endocrine and metabolic processes and in behavior.[12] Cotterell *et al*. identified specific binding sites for both α_1- and α_2-adrenoceptors in rat jejunal epithelial cell membranes and suggested that α_1-adrenoceptors predominate in this tissue and modulate absorptive processes, while α_2-adrenoceptors are involved in secretion.[13] The study of the distribution of the α_2-adrenergic receptor within duodenal and colonic mucosa showed that the receptor is preferentially expressed in crypt cells of humans.[14] It was suggested that norepinephrine stimulates epithelial cell proliferation in both small and large intestines and that this effect is mediated by an α_2-adrenoceptor located in crypt cells. In contrast, stimulation of α_1-and β-adrenoceptors is inhibitory to cell proliferation.[15,16] It was also suggested α_2 -adrenoceptors activate the MAPK pathway and act as co-mitogens in human intestinal epithelial cells.[17]

Norepinephrine-induced apoptosis has been studied in lymphocytes[18] and alveolar epithelia[19] mediated by α-, β-, and angiotensin receptor activation. Schauenstein *et al*. have shown that quiescent lymphocytes express the genes for α_1- and α_2- as well as β_2-receptors.[20] They considered that α-adrenergic treatment directly affects leukocyte functions, including cell adhesion, mitosis, and apoptosis.[6]

In our study, it was shown that norepinephrine increased radiation-induced apoptosis in IEC-18 cells. The blockage of norepinephrine by an adrenoceptor antagonist may result in decrease of radiation-induced apoptosis in normal intestinal epithelial cells. Previously we found that chemical sympathectomy with reserpine suppressed the radiation-induced apoptotic level in jejunal crypt cells of rats.[1] Zelefsky *et al*. suggested that acute urinary symptoms experienced during a course of radiotherapy can be effectively treated with selective α-blocker agents.[21] It has been suggested that α_1-adrenoceptor antagonists enhance radiation-induced apoptosis in human prostate cancer cells, and α_1-adrenoceptor antagonists in combination with radiotherapy can be used for the treatment of prostate cancer via an enhanced apoptotic response.[22] In our study it was not clear which receptor mediates the decrease of cell survival after irradiation or

induction of radiation-induced apoptosis in IEC-18 cells. Further studies will need to be carried out to determine which receptor is associated with high radiation sensitivity of NE treated cells.

In conclusion, this is the first study to demonstrate the enhancement of radiosensitivity by NE of rat ileal epithelial cells in vitro.

ACKNOWLEDGEMENTS

The study was supported by a grant from the Ministry of Education, Science, Sports and Culture of Japan for Young Scientists.

REFERENCES

1. Matsuu M, *et al* (2000) The role of the sympathetic nervous system in radiation-induced apoptosis in jejunal crypt cells of spontaneously hypertensive rats. J Radiat Res **41**: 55–65.
2. Matsuu M, *et al* (2005) Sympathetic hyperfunction causes increased sensitivity of the autonomic nervous system to whole-body X irradiation. Radiat Res **163**: 137–143.
3. Tsukada F, *et al* (2002) Mechanism of inhibition of small intestinal motility by restraint stress differs from that with norepinephrine treatment in rats. Biol Pharm Bull **25**: 122–124.
4. Chang EB, Fedrak RN and Field M (1986) Experimental diabetic diarrhea in rats. Intestinal mucosal denervation hypersensitivity and treatment with clonidine. Gastroenterology **91**: 564–569.
5. Straub RH, *et al* (2006) The role of the sympathetic nervous system in intestinal inflammation. Gut **55**: 1640–1649.
6. Stevenson JR, *et al* (2001) Prolonged alpha-adrenergic stimulation causes changes in leukocyte distribution and lymphocyte apoptosis in the rat. J Neuroimmunol **120**: 50–57.
7. Communal C, *et al* (1998) Norepinephrine stimulates apoptosis in adult rat ventricular myocytes by activation of the beta-adrenergic pathway. Circulation **98**: 1329–1334.
8. Fu YC, *et al* (2004) Norepinephrine induces apoptosis in neonatal rat endothelial cells via down-regulation of Bcl-2 and activation of beta-adrenergic and caspase-2 pathway. Cardiovascular Research **61**: 143–151.
9. Quaroni A, *et al* (1979) Epithelioid cell cultures from rat small intestine. Characterization by morphologic and immunologic criteria. J Cell Biol **80**: 248–265.
10. Chiu T, Santiskulvong C and Rozengurt E (2003) ANG II stimulates PKC-dependent ERK activation, DNA synthesis, and cell division in intestinal epithelial cells. Am J Physiol Gastrointest Liver Physiol **285**: G1–G11.
11. Uemura T, *et al* (2007) The protective effect of interleukin-11 on the cell death induced by X-ray irradiation in cultured intestinal epithelial cell. J Radiat Res **48**: 171–177.
12. Docherty JR (1998) Subtypes of functional α_1- and α_2-adrenoceptors. Eur J Pharmacol **361**: 1–15.
13. Cotterell DJ, Munday KA and Poat JA (1984) The binding of [^{3}H]prazosin and [^{3}H]clonidine to rat jejunal epithelial cell membranes. Biochemical Pharmacology **33**: 751–756.
14. Valet P, *et al* (1993) Characterization and distribution of α2-adrenergic receptors in the human intestinal mucosa. J Clin Invest **91**: 2049–2057.
15. Tutton PJM and Barkla DH (1989) Effect of an inhibitor of noradrenaline uptake, desipramine, on cell proliferation in the intestinal crypt epithelium. Virchows Archiv B Cell Pathol **57**: 349–352.
16. Kennedy MFG, Tutton PJM and Barkla DH (1983) Adrenergic factors involved in the control of crypt cell proliferation in jejunum and descending colon of mouse. Clinical and Experimental Pharmacology & Physiology **10**: 577–586.
17. Schaak S, *et al* (2000) Alpha$_2$ adrenoceptors regulate proliferation of human intestinal epithelial cells. Gut **47**: 242–250
18. Josefsson E, *et al* (1996) Catecholamines are synthesized by mouse lymphocytes and regulate function of these cells by induction of apoptosis. Immunology **88**: 140–146.
19. Dincer HE, *et al* (2001) Norepinephrine induces alveolar epithelial apoptosis mediated by α-, β-, and angiotensin receptor activation. Am J Physiol Lung Cell Mol Physiol **281**: L624–L630.
20. Schauenstein K, *et al* (2000) In vivo immunomodulation by peripheral adrenergic and cholinergic agonists/antagonists in rat and mouse models. Ann N Y Acad Sci **917**: 618–627.
21. Zelefsky MJ, *et al* (1999) Efficacy of selective alpha-1 blocker therapy in the treatment of acute urinary symptoms during radiotherapy for localized prostate cancer. Int J Radiation Oncology Biol Phys **45**: 567–570.
22. Cuellar DC, Rhee J and Kyprianou N (2002) Alpha1-adrenoceptor antagonists radiosensitize prostate cancer cells via apoptosis induction. Anticancer Res **22**: 1673–1680.

Received on October 28, 2010
Revision received on December 27, 2010
Accepted on January 7, 2011
J-STAGE Advance Publication Date: February 19, 2011