

# Induction of Apoptosis on K562 Cell Line and Double Strand Breaks on Colon Cancer Cell Line Expressing High Affinity Receptor for Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF)

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## ABSTRACT

**Background:** Immunotoxins are comprised of both the cell targeting and the cell killing moieties. We previously established a new immunotoxin, i.e. Shiga toxin granulocyte macrophage-colony stimulating factor (StxA1-GM-CSF), comprises of catalytic domain of Stx, as a killing moiety and GM-CSF, as a cell targeting moiety. In this study, the ability of the immunotoxin to induce apoptosis and double strand breaks (DSB) on different cell lines was investigated. **Methods:** The recombinant hybrid protein was expressed in bacterial expression system and purified with nickel-nitrilotriacetate acid resin. The K562 (erythroid leukemia) cell line and LS174 (colon carcinoma) were used in this study. The neutral comet assay was carried out for the detection of DSB and Hoechst staining was performed for apoptosis. **Results:** StxA1-GM-CSF effectively induced apoptosis on K562 cell line and DNA Double Strand Break (DSB) were observed on colon cancer cell line treated with StxA1-GM-CSF. **Conclusion:** This novel action i.e. DNA damage might be a relevant mechanism of action for StxA1-GM-CSF that is designed to act as immunotoxin, although further investigation is required. *Iran. Biomed. J. 12 (1): 1-6, 2008*

**Keywords:** Shiga toxin granulocyte macrophage-colony stimulating factor (StxA1-GM-CSF), Cancer cells, Apoptosis, Double strand breaks (DSB)

## INTRODUCTION

Most cancer cells are resistant to anticancer agents; one of the reasons is related to defect in induction of apoptosis because of expression of anti-apoptotic genes. So, induction of apoptosis through anticancer agents would, at least for some cancer cells, overcome the resistance. In the last few years, a new approach for targeted therapy of human disease has been developed using cytotoxic molecules that are produced by gene fusion techniques [1, 2]. This class of molecules termed chimeric proteins comprises both the cell targeting and the cell killing moieties. As killing moieties, bacterial or plant toxins are used. Shiga

toxin (Stx) and Shiga-like toxin (SLT) are ribosome-inactivating proteins (RIP) produced by *Shigella* and *E. coli*, composed of an enzymatic A subunit non-covalently associated with a pentamer receptor-binding subunit. SLT inhibit protein synthesis in eukaryotic cells by releasing an adenine residue from the highly conserved aminoacyl-tRNA-binding site which exists on large subunit of ribosome [1]. Human granulocyte-macrophage colony-stimulating factor (hGM-CSF) is a cytokine, required for production of granulocytes and macrophages from normal bone marrow and appears to regulate the activity of mature, differentiated granulocytes and macrophages. The receptor for this cytokine (GM-CSF [granulocyte macrophage-colony

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stimulating factor] receptor) is expressed as a heteromeric complex containing a  $\alpha$  and a  $\beta$  subunit. Such receptors with a narrow tissue distribution and a high affinity are promising targets to selectively deliver toxins to malignant cells. Furthermore, it has been shown that the GM-CSF receptor is probably not expressed by the phenotypically most immature subsets of CD34<sup>+</sup> cells [3, 4] and mRNA for this receptor was not detected in bone marrow cells, suggesting that normal hemopoietic stem cells may escape cell death induced by targeted toxins. In the previous studies, we established a new immunotoxins StxA1-GM-CSF and showed its cytotoxicity effect on various cell lines and also showed the apoptotic effect on colon cancer cell line [5, 6]. In the present study, apoptotic effect of recombinant hybrid protein on K562 cell line expressing high affinity receptors was investigated. We also have studied the ability of our immunotoxin to induce double strand breaks (DSB) on colon cancer cell line.

## MATERIALS AND METHODS

### *Preparation of purified StxA1-GM-CSF protein.*

The catalytic domain of Stx, StxA1, was fused to hGM-CSF by PCR. The recombinant protein was expressed in *E. coli* [5]. The expressed protein was purified by ProBond purification system kit (Invitrogen, USA) according to manufacturer's protocol. The amount of protein was estimated by protein assay kit (BIO-RAD, USA).

**Cell culture.** K562 (erythroid leukemia) and LS174 (colon carcinoma) were obtained from Cell Resource Center for Biomedical Research, IDAC, Tohoku University, Japan. These cell lines were grown in RPMI-1640 medium (Gibco-BRL, Germany) with 10% fetal bovine serum (Gibco-BRL, Germany).

**Comet assay.** The neutral comet assay was carried out according to Olive *et al.* [7] with some modifications. Briefly, freshly prepared cell suspension ( $2 \times 10^3/10 \mu\text{l}$ ) was mixed with 150  $\mu\text{l}$  of 1% low-melting agarose. The mixture was layered on top of the microscope slide coated by 1% agarose. After low melting agarose was solidified in a refrigerator, the slide was gently immersed in a freshly prepared lysing solution (2% SDS, 0.03M EDTA) for 30 min protected from light. After washing the slides with TBE buffer, the

electrophoresis was carried out at the condition of 25 V for 25 min. Then, the comets were visualized with 1 mM propidium iodide and analyzed under the fluorescent microscope.

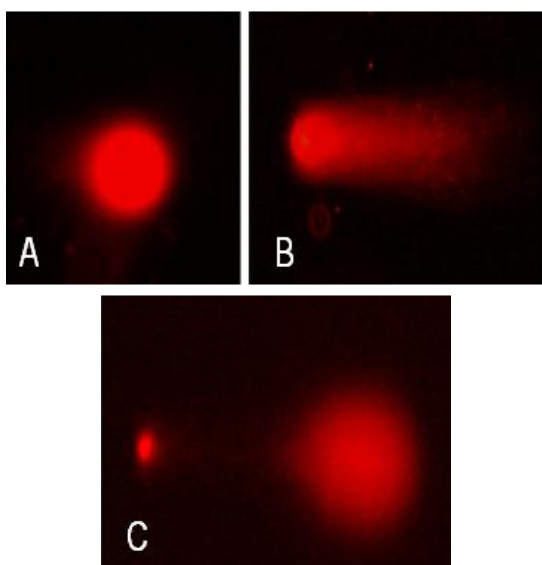
**Intracellular staining of gamma-H2AX.** To analyze the phosphorylation of gamma-H2AX,  $1 \times 10^2$  cells were cultured on the cover glass ( $1 \times 1 \text{ cm}$ ) for 24 hours. After the treatment, cells were fixed with 4% paraformaldehyde (Wako, Japan) for 15 min, washed, and permeabilized in 0.1% Triton X-100 (Sigma, Japan). Nonspecific binding was blocked by incubating with BSA at 4°C for 1.5 h. After the blocking step, cells were incubated with anti-gamma H2AX rabbit polyclonal antibody (1:100) (TREVIGEN, USA) at 4°C for 2 h. After washing with PBS, cells were incubated with FITC-conjugated anti-rabbit secondary antibody (1:1000) (DAKO, Denmark). Slides were mounted using Vectashield mounting medium for immunofluorescence. Spinals were observed under the Olympus AX70 fluorescent microscope (Olympus, Japan).

**Hoechst staining.** After administration of recombinant protein, cells were fixed with 1% paraformaldehyde for 30 min, washed with PBS and stained with 1 mM Hoechst 33342 (Sigma, Japan) for 10 min. Nuclear morphology of at least 400 cells was randomly observed under the fluorescent microscope.

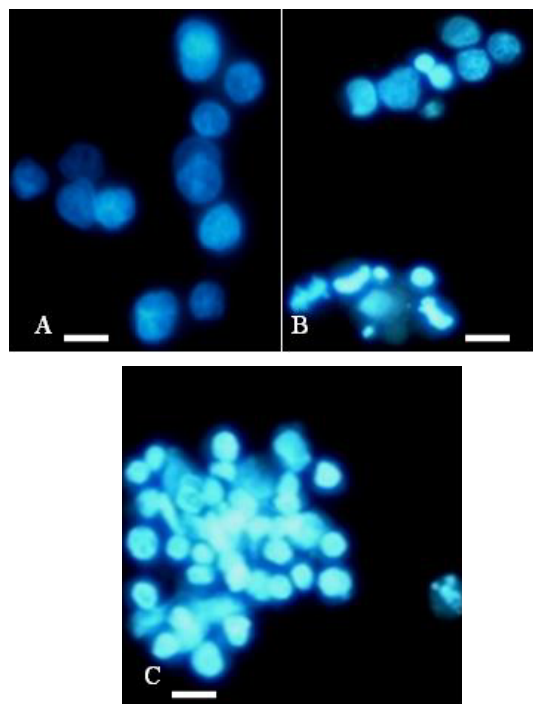
**Statistical analysis.** The results are expressed as mean  $\pm$  SD of three independent experiments. Differences among groups were compared using student's *t*-test ( $P < 0.05$ ,  $P < 0.01$  and  $P < 0.001$ ).

## RESULTS

**Apoptosis.** Comet assay and nuclear staining were used for assessment of the apoptotic effect of recombinant hybrid protein. In comet assay, apoptotic cells after treatment showed puffy tail and pin head while normal cells were spherical head and no tail (Fig. 1). To verify apoptotic results obtained by comet assay, cells treated in the same condition as in comet assay were analyzed with nuclear staining. Staining of cells with Hoechst 33342 after treatment with the fusion toxin showed condensed, small and fragmented appearance (Fig. 2). K562 cell line showed the characteristic of apoptotic cell in dose- and time-dependent manner (Figs. 3 and 4).



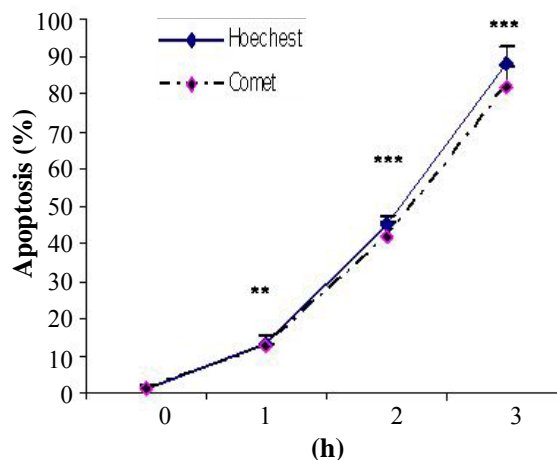
**Fig. 1.** Comet assay (A) control representing a normal cell and treated with hybrid protein exhibiting DSB (B) and apoptosis (C). Apoptotic cell represents puffy tail and pin head while cell containing DSB represent characteristics different from that of apoptosis (No pin head or puffy tail). We optimized time and concentration of hybrid protein in which the percentage of apoptosis was low. Apoptosis (C) related to K562 and DSB (B) related to LS174T cell line.



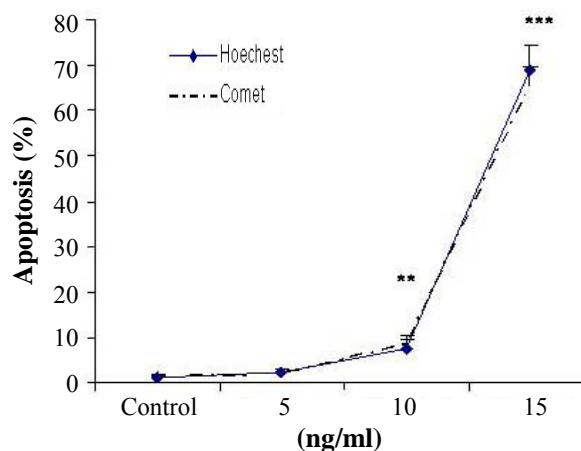
**Fig.2.** Hoechst 33342-staining of K562 cells treated with 40 ng/ml of StxA1-GM-CSF. (A) Control, (B) and (C) cells after treatment at 1 h and 3 h. Apoptotic cells are characterized with dense, small and fragmented nuclear in comparison to control.

The maximum apoptosis was observed in presence of 40 ng/ml and 3 h.

**Double strand breaks.** To determine whether StxA1-GM-CSF can induce DNA DSB on LS174T cell line, we used neutral comet assay. We optimized time and concentration of hybrid protein in which the cytotoxicity percentage were low. Damaged cell represent characteristic of comet containing tail but different with those observe in apoptotic (Fig. 1B).

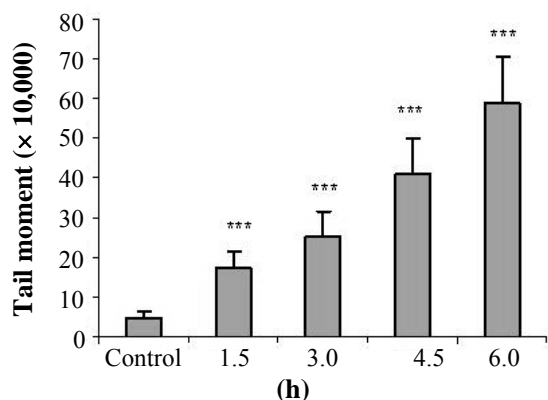


**Fig. 3.** Apoptosis percentage in the presence of 40 ng/ml StxA1-GM-CSF on K562 cell line at different time (mean  $\pm$  SD;  $P < 0.01$ ;  $P < 0.001$ ).



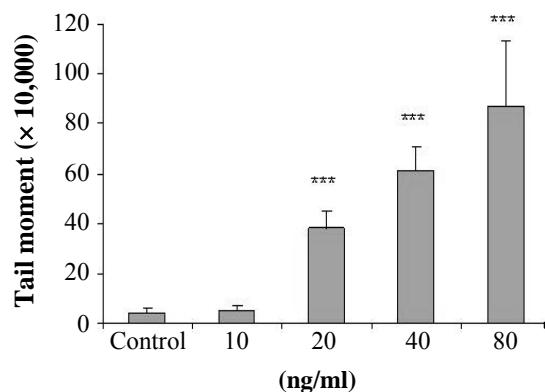
**Fig. 4.** Apoptosis percentage in the presence of different concentration of StxA1-GM-CSF on K562 cell line for 6 h (mean  $\pm$  SD;  $P < 0.01$ ;  $P < 0.001$ ).

The damage to double stranded DNA was measured by tail movement. Tail movement is defined as the product of the tail length and the fraction of total DNA in the tail. Tail movement



**Fig. 5.** Tail movement in the presence of 20 ng/ml StxA1-GM-CSF on LS174T cell line at different time (mean  $\pm$  SD; \*\*\*,  $P < 0.001$ ).

incorporates a measure of both the smallest detectable size of migrating DNA (reflected in the comet tail length) and the number of relaxed/broken pieces (represented by the intensity of DNA in the tail) [8-10]. The increase in percentage with time and concentration is suggestive of DSB and lack of DNA repairs (Figs. 5 and 6). DSB was also verified by gamma-H2AX staining. We found foci of phosphorylated histone gamma-H2AX after treatment of LS174T cells with StxA1-GM-CSF (Fig. 7).



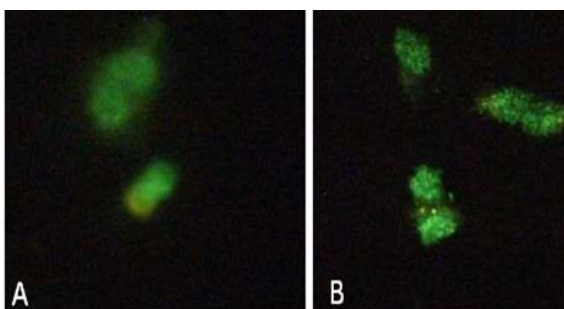
**Fig. 6.** Tail movement in the presence of different concentration of StxA1-GM-CSF on LS174T cell line for 4 h (mean  $\pm$  SD; \*\*\*,  $P < 0.001$ )

## DISCUSSION

In present study, we investigated the ability of recombinant hybrid protein, StxA1-GM-CSF to induce apoptosis on the K562 cell line. Frankle *et al.* [11] showed that DT-GM-CSF was ineffective on K562 in terms of cytotoxicity and apoptosis but

StxA1-GM-CSF was effective. We used comet assay [12] for detection of apoptosis. Usually, this method is used to detect DNA damage like single or DSB [8, 9]. On the other hand, this method also can detect single apoptotic cells in a large number of cells [12].

Death of cells treated with RIP or RIP containing fusion toxins was thought to be due to protein synthesis arrest and consequently necrosis. However, it was found that the morphology of cells treated with RIP resembled that of cells undergoing death by apoptosis [13]. It is logical to assume that StxA1-GM-CSF dose not activate the cellular



**Fig. 7.** Intracellular staining of gamma-H2AX. (A) Control, (B) induction of foci in the presence of 80 ng/ml StxA1-GM-CSF on LS174T cell line for 4 h.

apoptosis machinery directly as it is a primary activity of the toxin is to inactivate ribosomes and inhibit protein synthesis in the cells. Induction of apoptosis by Stx has been suggested but few studies have tried to delineate the mechanism of apoptotic signal pathway, although a critical role for caspase-3 has been proposed [13].

Since the potential application of RIP toxins lies in their targeted delivery in cancer immunotherapy, it is imperative that a systematic study to be carried out on various cancer lines to determine their efficacy in inducing apoptosis [14]. It is desirable that toxin mediated therapy of cancers induces apoptotic cell death and not necrosis, since necrosis results in the spillage of cellular content to extra cellular milieu, thereby triggering inflammatory response, which can be deleterious.

Overall our result showed StxA1-GM-CSF effectively induces apoptosis on haematopoietic cancer cell line, i.e. K562. We also investigated induction of DSB by StxA1-GM-CSF in colon cancer cell line, i.e. LS174T. We previously showed that our recombinant hybrid protein induced apoptosis on LS174T [6]. DSB was measured by neutral comet assay [7] and verified by immunocytochemistry of H2AX. Recently, the

phosphorylated form of histone variant H2AX (termed H2AX) has gained attention for its relationship with DNA damage, particularly DSB [15]. It has been shown that phosphorylation of H2AX forms localized “foci” at the sites of DSB [15, 16]. DSB are considered the most biologically important lesions, primary because of close relationship with chromosomal damage, which in turn is closely linked to cell death [17]. For more than almost two decades it has largely been assumed that RIP act only on rRNA within ribosomes [18, 19]. Recently, it was shown that all plants RIP and Stx tested are also able to remove several adenine residues from nicked DNA [20-25].

The novel phenomenon of DSB observed by StxA1-GM-CSF in our study could be due to the fact that internalized Stx is known to reach the nuclear envelope [26] and cells treated with Stx show the toxin predominantly in nuclear fraction [27]. More recently Sestili *et al.* [27], showed that StxA1 and ricin can induce SSB and inhibit the repair of DNA. Catalytic domain of Stx removes adenine from DNA and causes Single Strand Break (SSB) but because of inhibition of DNA repair it remains unpaired [27, 28]. Weakening of DNA sugar-phosphate backbone after extensive adenine removal [21, 24] and activation of endonucleases are the consequences of the activation for apoptotic cell death [29, 30]. Therefore, it seems reasonable to hypothesize that hybrid protein due to its catalytic domain enters the cells and directly challenges the integrity of nuclear DNA and induces DSB. However further studies are required to clarify its mechanism.

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#### REFERENCES

1. Stirpe, F. (2004) Ribosome-inactivating proteins. *Toxicon* 44: 371-381.
2. Reiter, Y. (2001) Recombinant immunotoxins in targeted cancer cell therapy. *Adv. Cancer Res.* 81: 93-124.
3. Chiba, S., Shibuya, K., Piao, Y., Tojo, A., Sasaki, N., Matsuki, S., Miyagawa, K. and Miyazono K.F. (1990) Identification and cellular distribution of distinct proteins forming human GM-CSF receptor. *Cell Regul.* 1: 327-335.
4. Wognum, A.W., Westerman, Y., Visser, T.P and Wagemaker, G. (1994) Distribution of receptors for granulocyte-macrophage colony-simulating factor on immature CD34+ bone marrow cells, differentiating monomyeloid progenitors, and mature blood cell subsets. *Blood* 84: 764-774.
5. Roudkenar, M.H., Bouzari, S., Oloomi, M., Jafari, A., Shahrokhi, N. and Shokrgozar, M.A. (2005) Expression of a chimeric protein containing the catalytic domain of Shiga-like toxin and human granulocyte-macrophage colony-stimulating factor (hGM-CSF) in *Escherichia coli* and its recognition by reciprocal antibodies. *Iran. Biomed. J.* 9: 143-148.
6. Roudkenar, M.H., Bouzari, S., Kuwahara, Y., Roushandeh, M.A., Oloomi, M. and Fukumoto, M. (2006) Recombinant hybrid protein, Shiga toxin and granulocyte macrophage colony stimulating factor effectively induce apoptosis of colon cancer cells. *World J. Gastroenterol.* 12: 2341-2344.
7. Olive, P.L., Wlodek, D. and Banata, J. (1991) DNA Double-strand breaks in individual cells subjected to gel electrophoresis. *Cancer Res.* 51: 4671-4676.
8. Tada, H., Shiho, O., Kuroshima, K., Koyama, M. and Tsukamoto, K. (1986) An improved colorimetric assay for interleukin 2. *J. Immunol. Methods* 9: 157-165.
9. Collins, A.R. (2004) The comet assay for DNA damage and repair: principles, applications, and limitations. *Mol. Biotechnol.* 26: 249-261.
10. Lee, R.F. and Steinert, S. (2003) Use of the single cell gel electrophoresis/comet assay for detecting DNA damage in aquatic (marine and freshwater) animals. *Mutat. Res.* 544: 43-64.
11. Frankel, A.E., Hall, P.D., Burbage, C., Vesely, J., Willingham, M., Bhalla, K. and Kreitman, R.J. (1997) Modulation of the apoptotic response of human myeloid leukemia cells of a diphtheria toxin granulocyte-macrophage colony-stimulating factor fusion protein. *Blood* 90: 3654-3661.
12. Singh, N.P. (2000) A simple method for accurate estimation of apoptotic cells. *Exp. Cell Res.* 256: 328-337.
13. Narayanan, S., Surendranath, K., Bora, N., Surolia, A. and Karande, A.A. (2005) Ribosome inactivating protein and apoptosis. *FEBS Lett.* 579: 1324-1331.
14. Lee, S.Y., Cherla R.P. and Tesh, V.L. (2007) Simultaneous induction of apoptotic and survival signaling pathways in macrophage-like THP-1 cells by Shiga toxin 1. *Infect. Immun.* 75: 1291-302.
15. Pilch, D.R., Sedelnikova, O.A., Redon, C., Celeste, A., Nussenzweig, A. and Bonner, M.W. (2003) Characteristics of gamma-H2AX foci at DNA double-strand breaks sites. *Biochem. Cell Biol.* 81: 123-129.
16. Rogakou, E.P., Pilch, D.R., Orr, A.H., Ivanova, V.S. and Bonner, W.M. (1998) DNA double-stranded breaks induce histone H2AX phosphorylation on

- serine 139. *J. Biol. Chem.* 273: 5858-5868.
17. MacPhail, S.H., Banath, J.P., Yu, T.Y., Chu, E.H., Lambur, H. and Olive, P.L. (2003) Expression of phosphorylated histone H2AX in cultured cell lines following exposure to X-rays. *Int. J. Radiat. Biol.* 79: 351-358.
  18. McMillan, T.J., Tobi, S., Mateos, S. and lemon, C. (2001) The use of DNA double-strand break quantification in radiotherapy. *Int. J. Radiat. Oncol. Biol. Phys.* 49: 373-377.
  19. Endo, Y. and Tsurugi, K.R. (1987) RNA N-glycosidase activity of ricin A-chain. Mechanism of action of the toxic lectin ricin on eukaryotic ribosomes. *J. Biol. Chem.* 262: 8128-8130.
  20. Endo, Y., Tsurugi, K., Yutsudo, T., Takeda, Y., Ogasawara, T. and Igarashi, K. (1988) Site of action of a Vero toxin (VT2) from *Escherichia coli* O157:H7 and of Shiga toxin on eukaryotic ribosomes. RNA N-glycosidase activity of the toxins. *Eur. J. Biochem.* 171: 45-50.
  21. Barbicri, L., Gorini, P., Valbonesi, P. and Stirpe, F. (1994) Unexpected activity of saporins. *Nature (London)* 372: 624.
  22. Barbicri, L., Valbonesi, P., Bonora, P., Gorini, P., Bolognesi, A. and Stirpe, F. (1997) Polynucleotide: adenosine glycosidase activity of ribosome-inactivating proteins: effect on DNA, RNA and poly (A). *Nucleic Acids Res.* 25: 518-522.
  23. Barbicri, L., Valbonesi, P., Brigotti, M., Montanaro, L., Stirpe, F. and Sperti, S. (1998) Shiga-like toxin I is a polynucleotide: adenosine glycosidase. *Mol. Microbiol.* 29: 661-662.
  24. Brigotti, M., Barbicri, L., Valbonesi, P., Stirpe, F., Montanaro, L. and Sperti, S. (1998) A rapid and sensitive method to measure the enzymatic activity of ribosome-inactivating proteins. *Nucleic Acids Res.* 26: 4306-4307.
  25. Nicolas, E., Beggs, J.M. and Taraschi, T.F. (2000) Gelonin is an unusual DNA glycosylase that removes adenine from single-stranded DNA, normal base pairs and mismatches. *J. Biol. Chem.* 275: 31399-31406.
  26. Sandvig, K., Garred, O., Prydz, K., Kozlov, J.V., Hansen, S.H. and Van Deurs, B. (1992) Retrograde transport of endocytosed Shiga toxin to the endoplasmic reticulum. *Nature (London)* 358: 510-512.
  27. Sestili, P., Alfieri, R., Carnicelli, D., Martinelli, C.B., Arbieri, D., Stripe, F., Bonelli, M., Pier, G.F. and Brigotti, M. (2005) Shiga toxin 1 and ricin inhibit the repair of H<sub>2</sub>O<sub>2</sub>- induced DNA single strand breaks in cultured mammalian cells. *DNA Repair* 4: 271-277.
  28. Suzuki, A., Doi, H., Matsuzawa, F., Aikawa, S., Takiguchi, K., Kawano, K., Hayashida, M. and Ohno, S. (2000) Bcl-2 anti-apoptotic protein mediates verotoxin II-induced cell death: possible association between Bcl-2 and tissue failure by *E. coli* O157:H7. *Genes Dev.* 14: 1734-1740.
  29. Brigotti, M., Alfieri, R., Sestili, P., Bonelli, M., Petronini, P.G., Guidarelli, A., Barbieri, L., Stirpe, F. and Sperti, S. (2002) Damage to nuclear DNA induced by Shiga toxin 1 and ricin in human endothelial cells. *FASEB J.* 16: 365-372.
  30. Dodson, M.L., Michaels, M.L. and Lloyd, R.S. (1994) Unified catalytic mechanism for DNA glycosylase. *J. Biol. Chem.* 269: 32709-32712.