Interferon-Alpha and Interferon-Gamma Augment Apoptosis in Oral Carcinoma Cells Treated with Shiga Toxin 2 (Stx2)

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ABSTRACT

It has been reported that shiga toxin 1 (Stx1) or shiga toxin 2 (Stx2) produced by enterohemorrhagic Escherichia coli (EHEC) induces apoptosis in several cell lines including ACHN (human renal adenocarcinoma cell line), Vero (african green monkey kidney cell line), MDCK (madin darby canine kidney cell line), human B lymphoid cell lines, human astrocytoma cell lines, human breast cancer cell lines and human renal tubular epithelial cells. Apoptosis mediated by shiga toxin 2 has been identified in several human oral carcinoma cell lines, OSC-19, OSC-20, OSC-30, OSC-40 and OSC-70, in our laboratory. These cell lines were positive in the expression of receptor molecule Gb3/CD77 recognized by the toxin. Cell death by Stx2, however, was not correlated with the expression levels of CD77 confirmed by positive staining with antibody to the receptor molecule. Pretreatment of cells with interferon-\(\alpha\) (IFN-\(\alpha\)) or IFN-\(\gamma\) augmented apoptosis induced by the Stx2. Sensitivity of these cells to IFN-\(\alpha\) and IFN-\(\gamma\) was confirmed by induction of 2',5'-oligoadenylate synthetase or interferon regulatory factor-1. Even after treatment with IFNs, expression of CD77 did not fluctuate. Therefore, augmentation of apoptosis by IFNs was not correlated with the increase in expression levels of receptor to the toxin. IFN-\(\gamma\) has the ability to induce and significantly augment cell death of these carcinoma cells in comparison with IFN-\(\alpha\).

Key words: Interferon, Apoptosis, Oral cancer, Shiga-like toxin

INTRODUCTION

Enterohemorrhagic Escherichia coli (EHEC) is an etiologic promote agent of outbreaks of hemorrhagic colitis and the hemolytic-uremic syndrome\(^{3-5}\). The strains of EHEC produce potent cytotoxins termed shiga-like toxins or verotoxins. The toxins consist of two antigenically distinct subunits, A and B. The A subunit is associated with inhibition of cellular protein synthesis by catalytically inactivating the 60S ribosomal subunit, and the B subunit of pentamer is responsible for the binding of the toxin to its receptor (CD77/Gb3)\(^{5-7}\). It is well known that shiga toxin types 1 and 2 (Stx 1 and Stx2) induce cell death characteristic of apoptosis in several cell lines including Vero.

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MDCK, ACHN, human astrocytoma and Burkitt's lymphoma. There is evidence that induction of apoptosis is caused by inhibition of protein synthesis. The A subunit is, therefore, essential for induction of apoptosis in Vero cells through inhibition of protein synthesis. Furthermore, cycloheximide, an inhibitor of protein synthesis can augment Stx-mediated apoptosis in ACHN cells, though the mechanism for this is still unknown. Recent reports describing interferon (IFN) induced or enhanced apoptosis may support the hypothesis that inhibition of protein synthesis increases the possibility of induction of a self-destructive process of cell death. Indeed, apoptosis induced by IFN is thought to be mediated through activation of IFN-signaling pathway to produce both PKR (ds-RNA activated protein kinase) and 2-5AS (2',5'- oligoadenylate synthetase)/RNase L system, and inhibits protein synthesis. Recently, we reported that IFN-α can enhance Stx2-induced apoptosis in lymphoma cell lines. The augmentation is thought to be associated with inhibition of protein synthesis.

As described herein, the shiga toxins are active against various tumor cell lines in vitro and in vivo. In this report, we extend the spectrum of anti-tumor activity of Stx2 to human oral carcinoma cell lines, and demonstrate the augmentation of Stx2-induced apoptosis by IFN.

MATERIALS AND METHODS

Cell lines

Properties of human squamous carcinoma cell lines used in this experiment are summarized in Table 1. These cell lines were established from a metastatic oral squamous carcinoma by the same procedures as described previously, and cultured with RPMI 1640 medium supplemented with 10% heat-inactivated foetal bovine serum and 100 units/ml of penicillin in a humidified 5% CO2 incubator at 37 C.

<table>
<thead>
<tr>
<th>Cell</th>
<th>Tumor location</th>
<th>Histology of primary tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>OSC-19</td>
<td>Tongue</td>
<td>Moderately diff. sq.</td>
</tr>
<tr>
<td>OSC-20</td>
<td>Tongue</td>
<td>Well diff. sq.</td>
</tr>
<tr>
<td>OSC-30</td>
<td>Tongue</td>
<td>Moderately diff. sq.</td>
</tr>
<tr>
<td>OSC-40</td>
<td>Oral floor</td>
<td>Well diff. sq.</td>
</tr>
<tr>
<td>OSC-70</td>
<td>Tongue</td>
<td>Well diff. sq.</td>
</tr>
</tbody>
</table>

a: differentiated, b: squamous cell carcinoma.

IFN or Stx2 treatment and live cell percentage

Each cell line cultured at 80% confluence and showing the same absorbance of cell density was treated with 10^3 IU/ml of IFN-α (Sorotec Inc., Oxford, U.K.) or IFN-γ (Genzyme Corp., Cambridge, MA, USA) for 24 h, and then the cells were washed, and cultured for an additional 2 days with shiga toxin 2 (Stx2) purchased from Nacalai Tesque (Kyoto, Japan). They were then washed, stained with gentian violet solution for 2 or 3 min, washed again and dried at room temperature. The dye binding to the cells was dissolved with methyl cellulose for 3 h, diluted tenfold with the same solvent and absorbance was measured at 550 nm in accordance with the method described previously. The percentage of live cells in IFN or Stx2 treated cells was determined as a ratio in relation to untreated control cells.

DNA fragmentation assay

DNAs were extracted from the cells treated with or without Stx2, and the fragmented DNAs were visualized by agarose gel electrophoresis using UV light as described previously. To measure the extent of apoptosis, a cellular DNA fragmentation ELISA kit (Boehringer Mannheim, Indianapolis, IN, USA) was used according to the manufacturer's instructions. This assay is based on a photomeric ELISA for detection of BrdU-labeled DNA fragments in cell lysates.

Sensitivity of cells to IFN

Cells were cultured with 500 IU/ml of IFN-α or 10^3 IU/ml of IFN-γ for 24 h. To assess
their sensitivity to IFN-α, IFN-α induced 2',5'-oligoadenylate synthetase (2-5AS) activity was measured in cell lysates by using the same method as described previously\(^2^{29,30}\). IFN-γ induced STAT-1 (signal transducers and activators of transcription) and IRF-1 (interferon regulatory factor-1) inductions were investigated by western blot analysis in accordance with the method described in a previous report\(^31,32\). Antibodies for STAT-1 and IRF-1 were purchased from Transduction (Lexington, KY, USA) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), respectively.

**Flow cytometry**

After treatment of cells with 10^5 IU/ml of either IFN-α or IFN-γ or without IFNs for 24 h, the expression of Gb3/CD77 on the cell surface was determined by using an anti-CD77 monoclonal antibody (clone 38.13, IgM). Cells stained by FITC-labeled F(ab') fragment from anti-rat IgM antibody were analyzed with a FACScan (Becton Dickinson).

**RESULTS AND DISCUSSION**

**Apoptosis induced by Stx2 in human oral squamous carcinoma cell lines**

It has been reported that Stx2 induces apoptosis in several human tumor cells\(^8,9,11,12,15\). Human oral carcinoma cell lines, OSC-19, OSC-20, OSC-30, OSC-40 and OSC-70 treated with Stx2 were examined for induction of apoptosis/cell death by measuring gentian violet uptake and by DNA fragmentation assay. As shown in Fig. 1, Stx2 induced cell death after cultivation of cells with the toxin for 48 h. However, the susceptibility of the cell lines to the toxin was varied; OSC-20 and OSC-70 were highly sensitive to Stx2, but OSC-30 was resistant to the toxin. Cell death induced by Stx2 was dose dependent (Fig. 2). The different susceptibilities of the cell lines is thought to be, in part, caused by the level of expression of Gb3/CD77, because a large degree of cell death was observed in OSC-70 and OSC-20 cells with a high level of this receptor (Table 2). However, the expression of Gb3/CD77 in OSC-30 was almost of the same levels as those of OSC-19 and OSC-40. It is, therefore, a possibility that some factor other than Stx2 is also involved in the induction of apoptosis by Stx2. Furthermore, it is also suggested that cellular-resistant mechanisms to apoptosis/cell death induced by Stx2 is different depending on the cell types. More specifically OSC-30 cells are thought to be relatively
Table 2 Expression of CD77 in human oral carcinoma cell lines.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Medium</th>
<th>IFN-alpha</th>
<th>IFN-gamma</th>
</tr>
</thead>
<tbody>
<tr>
<td>OSC-19</td>
<td>28.1</td>
<td>30.2</td>
<td>26.6</td>
</tr>
<tr>
<td>OSC-20</td>
<td>42.4</td>
<td>45.7</td>
<td>40.2</td>
</tr>
<tr>
<td>OSC-30</td>
<td>26.7</td>
<td>38.4</td>
<td>32.4</td>
</tr>
<tr>
<td>OSC-40</td>
<td>30.5</td>
<td>28.2</td>
<td>30.0</td>
</tr>
<tr>
<td>OSC-70</td>
<td>71.8</td>
<td>68.2</td>
<td>69.3</td>
</tr>
</tbody>
</table>

a) The expression of CD77 on the cell membrane was determined by using an anti-CD77 mAb (clone 38.13, IgM). Cells stained by FITC-labeled F(ab')2 fragment from anti-rat IgM antibody were analyzed with a FACScan (Becton Dickinson).

b) Cells were treated with IFN for 24 h, and then the CD77-positive cells were determined by the same procedure as described above.

resistant to induction of apoptosis by Stx2. OSC-70 cells treated with Stx2 were examined for induction of apoptosis by using the DNA fragmentation assay and a fragmentation pattern characteristic of apoptosis was observed on agarose gel electrophoresis. The level of DNA fragmentation increased in accordance with cultivation time (Fig.3). Similar results were also obtained in other cell lines (data not shown).

Cell viabilities reached the minimum level in each cell line on the second day, and then the live cell numbers gradually increased. It was suggested that the cell populations with little expression of CD77 on their surface were alive, and that the CD77-negative cells grew in place of the cells killed by Stx2. Therefore, the cytotoxic effect of Stx2 on carcinoma cell lines is thought to be limited by the presence of CD77 although an exact correlation between the expression level of CD77 and the induction of cell death was not established (Fig.1 and Table 2). Induction of cell death may be dependent on an adequate amount of Stx2 binding to the cell membrane. Therefore, it is suggested that effective induction of apoptosis/cell death is not found in cell-populations carrying only a few receptors because of the small amount of bound Stx2. It has been reported that there is evidence for augmentation and induction of apoptosis by IFNs39–42. We examined that effect of IFNs on Stx2-induced apoptosis in oral carcinoma cell lines and this necessitated an investigation into the susceptibility of carcinoma cells to IFN-α and IFN-γ.

Susceptibility of cells to IFN

It is well known that IFNs have the ability to express various IFN-stimulated genes (ISG)34,35. Among them, 2',5'-oligoadenylate synthetase (2-5AS) and interferon regulatory factor-1 (IRF-1) are prominent markers for response to IFN-α and IFN-γ, respectively36–38. Susceptibility of oral carcinoma cell lines to IFNs was examined by induction of 2-5AS or IRF-1 after treatment with 10^6 IU/ml of IFNs. The activity of 2-5AS was significantly induced in these cell lines, with the exception of OSC-70 (Table 3). Although, only a two or three fold-induction of the enzyme activity was demonstrated in OSC-70 cells, constitutive production of 2-5AS was found in the cell line (Table 3). Induction of IRF-1 and STAT-1α was also recognized in these cell lines treated with IFN-γ (Fig.4). Con-
Table 3  Induction of 2',5'-oligoadenylyl synthetase (2'-5AS) by IFN-α

<table>
<thead>
<tr>
<th>Cells</th>
<th>2-5AS activity (nmole/mg·h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>OSC-19</td>
<td>6.3</td>
</tr>
<tr>
<td>OSC-20</td>
<td>4.4</td>
</tr>
<tr>
<td>OSC-30</td>
<td>16.3</td>
</tr>
<tr>
<td>OSC-40</td>
<td>20.4</td>
</tr>
<tr>
<td>OSC-70</td>
<td>26.6</td>
</tr>
</tbody>
</table>

Fig. 4  Western blot analysis for STAT-1α and IRF-1.

Cells were treated with 10⁶ IU/ml of IFN-γ for either 0 or 24 h, and then STAT-1α and IRF-1 in cell lysates were investigated by western blot analysis.

Supplementary production of STAT-1α was shown in OSC-40 and OSC-70 cells. STAT-1α is a key factor/component of IFN-signaling pathway for induction of 2-5AS or IRF-1. However, it is unclear whether endogenous activity of STAT-1α is correlated with spontaneous production of 2-5AS in OSC-40 and OSC-70 cells. Human oral carcinoma cell lines used in this experiment were significantly sensitive to both IFN-α and IFN-γ.

Augmentation of Stx2-induced apoptosis by IFNs

Recent studies on augmentation of Stx2-induced [23] or HPC (hexadecylphosphocholine)-induced apoptosis by IFNs [24] was reported in human B lymphoid cell lines or human leukemia cell lines. The effect of IFNs on enhancement of apoptosis is thought to be correlated with induction of 2-5AS or PKR through activation of IFN signaling pathway [24]. We examined the effect of IFNs on Stx2-induced apoptosis in human oral carcinoma cell lines. These cell lines were cultured with 10³ IU/ml of IFN-α or IFN-γ for 24 h, then washed and cultured with 64 pg/ml of Stx2 for an additional 2 days. The pretreatment of these cell lines with IFNs elicited augmentation of Stx2-induced cell death (Fig.5, A and B). Pretreatment of cells with IFN-γ was more effective at inducing apoptosis than with IFN-α. All of the cell lines died out

Fig. 5  Augmentation of Stx2-induced apoptosis by IFNs.

Pretreated cells with 10⁶ IU/ml of IFN-α (Panel A, □ and ■) or IFN-γ (panel B, □ and ■) for 24 h were washed, cultured either with [□] and ■) or without (□) 64 pg/ml of Stx2 for an additional 2 days. Cell viabilities were determined as the ratio against untreated control cell. Data were analyzed by Mann-Whitney’s U test. Combination with IFN-α and Stx2 (□) in panel A showed that cell cytotoxicity is highly significant (P<0.01) in OSC-19, OSC-30, and OSC-70 in comparison with that induced by only Stx2 (■) or IFN-α (□), but not in OSC-20 and OSC-40 (P>0.05). Combination with IFN-γ and Stx2 (■) in panel B showed that cell cytotoxicity is highly significant (P<0.01) in comparison with that induced by only Stx2 (□) or IFN-γ (□)
within a week following this treatment with IFN-γ and Stx2 (data not shown). Augmentation of Stx2-induced apoptosis by IFNs was confirmed by detection of DNA ladder formation on agarose gel electrophoresis (Fig.6). An accelerated DNA fragmentation pattern was most clearly found in the case of pretreatment with IFN-γ (Fig.6, lane 6), but a small amount of fragmentation could be observed with Stx2 alone (Fig.6, lane 4). The extent of apoptosis was also examined by using a cellular DNA fragmentation kit. As shown in Fig.7, DNA fragmentation was significantly induced by pretreatment with IFN-γ in OSC-30 and OSC-70 cell lines in comparison with Stx2 alone (Fig.7, columns 4 and 6). Apoptosis augmented by both IFNs was not caused by an increase in CD77 expression because the expression level was not affected (Table 2). The mechanism of augmentation of Stx2-induced apoptosis by IFNs remains to be elucidated. However, it is proposed that IFN-induced or IFN-enhanced apoptosis combines with activation of RNaseL or PKR to inhibit protein synthesis through IFN-signaling pathway. As STAT-1α is a key factor in the signaling, the strong susceptibility of OSC-70 to IFN is thought to result from constitutive productions of 2-5AS and STAT-1α. Inhibition of protein synthesis caused by Stx2 and IFNs might be essential for this event to take place. In other words, signaling pathway of apoptosis may be enhanced by treatment of the cell populations bound by a small amount of Stx2 with IFNs. On the other hand, recent studies revealed that apoptotic cell death induced by IFN-γ is regulated by activation of DAP (death associated protein) kinase, a new type of calcium/calmodulin-dependent enzyme, or expression of pro-apoptotic protein Bak\(^{40,41}\). It is, therefore, suggested that these factors are also involved in augmentation of Stx2-induced apoptosis by IFN-γ.

In contrast with the results described above, cell death induced by pretreatment with IFN-α or IFN-γ alone was also found in these cell lines (Fig. 5). IFN-γ induced cell death was more effective than that of IFN-α or stx2. However, a DNA fragmentation pattern characteristic of apoptosis was not recognized in cell-kill-

![Fig. 6 Augmentation of Stx2-induced DNA fragmentation by IFNs.](image)

OSC-30 cells were pretreated with 10⁵ IU/ml of IFN-α (lanes 2 and 5) or IFN-γ (lanes 3 and 6) for 24h, and then the cells were washed, treated either with (lanes 4, 5 and 6) or without (lanes 2 and 3) 64 pg/ml of Stx2 for an additional 2 days. Lane 1 is untreated control cells. M, molecular marker.

![Fig. 7 Extent of apoptosis by ELISA analysis of cell lysates](image)

OSC-30 (A) and OSC-70 (B) cells were pretreated with 10³ IU/ml of IFN-α (columns 2 and 5) or IFN-γ (columns 3 and 6) for 24 h, and then the cells were washed, treated either with (columns 4, 5 and 6) or without (columns 2 and 3) 64 pg/ml of Stx2 for an additional 2 days. Cells treated in the same way but with no IFNs and Stx2 added (untreated control cells) are indicated in column 1.
ing induced by IFNs (Fig. 6, lanes 2 and 3; Fig. 7, columns 2 and 3). It is still unknown whether the cell death induced by pretreatment with IFN is apoptosis. However, in general, cell killing systems are divided into two categories, necrosis and apoptosis. A killing process which occurs without DNA fragmentation is associated with necrotic cell death. IFN-γ induced cell death described herein was distinguishable from the process characterized by swelling and disintegration of cells and nuclei (data not shown). Therefore, it is proposed that IFN-γ raises the possibility of an unknown pathway by which cells undergo cell-killing. The mechanisms of cell death induced by IFN-γ alone should be analyzed in future studies.

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