Effects of Irradiation on Cytokine Production in Glioma Cell Lines

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Abstract

The effects of irradiation on cytokine production in glioma cell lines, NP1, NP2 and NP3, were studied. Culture supernatants were collected after 6, 24, 48 or 72 hours and the concentrations of interleukin (IL)-6 and IL-8 measured by enzyme-linked immunosorbent assay. Spontaneous and IL-1β-stimulated productions were analyzed. Some cells were given a single dose of Lineac irradiation (10 or 20 Gy).

Production of IL-6 (with or without IL-1β stimulation) increased gradually to a maximum after 72 hours, more in the 20 Gy-irradiated cells than 10 Gy cells (p < 0.01). Production of IL-8 increased gradually to a maximum after 48 or 72 hours. Spontaneous production of IL-8 increased more in 20 Gy-irradiated cells than 10 Gy cells after 6 and 24 hours (p < 0.01), but increased more in 10 Gy cells than 20 Gy cells after 48 and 72 hours (p < 0.01). The production of IL-8 stimulated by IL-1β increased more in 10 Gy cells than 20 Gy cells 24 hours later (p < 0.01). IL-6 and IL-8 production differed in the response to irradiation. Our data suggest that bidirectional communication between the immune system and glioma cells changes after radiotherapy.

Key words: glioma, interleukin-1β, interleukin-6, interleukin-8, radiation therapy

Introduction

There is increasing evidence for bidirectional communication between the immune and central nervous systems, especially mediated by soluble factors. Various human malignant glioma cell lines demonstrate similar immune-related responses, such as synthesis of interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)-α, granulocyte-macrophage colony stimulating factor (GM-CSF) and basic fibroblast growth factor, expression of human leukocyte antigen (HLA)-DR, and an increase in HLA-DR expression following stimulation with interferon (IFN)-γ. IL-1 is a cytokine with multiple biological actions in the regulation of the immune, inflammatory, endocrine and central nervous systems. Biological activities include the induction of various cytokines such as GM-CSF and IL-6.

Radiotherapy is generally used in patients with malignant glioma. Any irradiation effect on cytokine production in glioma cells would be interesting, as bidirectional communication between the immunoregulatory and glioma cells would presumably change. In this study, we investigated the effects of irradiation on the production of cytokines in human glioma cell lines.

Materials and Methods

I. Human glioma cell lines

Three human glioma cell lines, NP1, NP2 and NP3, were maintained in modified Eagle’s medium (MEM) supplemented with 10% fetal bovine serum (FBS) (Nissui, Tokyo). All media were free of endotoxin contamination (< 30 pg/ml).

II. Culture supernatants

Cell lines (1 x 10^5/ml) were grown in MEM-1% FBS in 24-well plates (Corning, New York, N.Y., U.S.A.) incubated for 48 hours at 37°C under 5% CO₂. The monolayer cells were washed three times with phosphate buffered saline, and MEM-1% FBS with or without 50 IU/ml of IL-1β (Otsuka, Tokyo) was added. The cells were cultured under the same conditions for 6, 24, 48 or 72 hours. The supernatants were then collected and stored at −80°C prior to assay.
III. Radiation

After the culture medium was replaced with fresh medium with or without IL-1β, some plates were exposed to a single dose of Lineac radiation (10 or 20 Gy). The supernatants of the culture medium were then collected as above. Almost all tumor cells remained viable for at least 72 hours.

IV. Measurement of cytokine production

Secretion of cytokines, IL-6 and IL-8, was measured using enzyme-linked immunosorbent assay with Quantikine D6000 and D8000 (R & D Systems, Inc., Minneapolis, Minn., U.S.A.). The optical density of each well at 450 nm was determined using a spectrophotometer. A standard curve was prepared using standard solutions. By comparing the optical density of the samples to this standard curve, the concentration of cytokines in the samples could be determined. All samples were assayed in triplicate. Data are presented as the mean value ± SEM. Student’s t-test was used to assess significance, with p < 0.05 considered statistically significant.

Results

I. Production of IL-6

Spontaneous production of IL-6 was about 10–1000 pg/ml. Production of IL-6 after stimulation by IL-1β was about 1000–80,000 pg/ml, both showing a gradual increase to a maximum after 72 hours (Tables 1 and 2). Spontaneous and IL-1β-stimulated productions were greater in the 20 Gy-irradiated cells than in the 10 Gy cells (p < 0.01) for all three cell lines.

Table 1 Spontaneous production of IL-6

<table>
<thead>
<tr>
<th>Hrs</th>
<th>NP1</th>
<th>NP2</th>
<th>NP3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-treated</td>
<td>10 Gy</td>
<td>20 Gy</td>
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</tbody>
</table>
| 6   | 15 ± 12 | 48 ± 21
|     | (6) | (9) | (6) | 96 ± 45
|     | (6) | (9) | (6) | 93 ± 32 | 8 ± 6 | 220 ± 43
|     | (6) | (9) | (6) | 24 ± 45 | 100 ± 14
|     | (6) | (9) | (6) | 110 ± 56

Values are means ± SEM (n = 3), expressed as pg/ml. *Significantly greater than non-treated: p < 0.01, bsignificantly greater than other irradiated value: p < 0.01.

Table 2 Production of IL-6 stimulated by IL-1β

<table>
<thead>
<tr>
<th>Hrs</th>
<th>NP1</th>
<th>NP2</th>
<th>NP3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-treated</td>
<td>10 Gy</td>
<td>20 Gy</td>
</tr>
</tbody>
</table>
| 6   | 2700 ± 125 | 4500 ± 280
|     | (6) | (9) | (6) | 4200 ± 350
|     | (6) | (9) | (6) | 1800 ± 500 | 170 ± 32 | 4400 ± 540
|     | (6) | (9) | (6) | 1000 ± 200 | 280 ± 50 | 2000 ± 260
| 24  | 60000 ± 850 | 50000 ± 880 | 92500 ± 760
|     | (6) | (9) | (6) | 2500 ± 700 | 2800 ± 450 | 10500 ± 790
|     | (6) | (9) | (6) | 3800 ± 460 | 2600 ± 560 | 5400 ± 670
| 48  | 65000 ± 640 | 60000 ± 900 | 90000 ± 900
|     | (6) | (9) | (6) | 6000 ± 980 | 5300 ± 670 | 18000 ± 300
|     | (6) | (9) | (6) | 4700 ± 650 | 4000 ± 460 | 9400 ± 780
| 72  | 80000 ± 900 | 75000 ± 860 | 92500 ± 1030
|     | (6) | (9) | (6) | 13000 ± 1200 | 10800 ± 990 | 35000 ± 2500
|     | (6) | (9) | (6) | 5400 ± 800 | 5600 ± 1400 | 14000 ± 1700

Values are means ± SEM (n = 3), expressed as pg/ml. *Significantly greater than non-treated: p < 0.01, bsignificantly greater than other irradiated value: p < 0.01.

Table 3 Spontaneous production of IL-8

<table>
<thead>
<tr>
<th>Hrs</th>
<th>NP1</th>
<th>NP2</th>
<th>NP3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-treated</td>
<td>10 Gy</td>
<td>20 Gy</td>
</tr>
</tbody>
</table>
| 6   | 3.1 ± 0.75 | 2 ± 0.4
|     | (6) | (9) | (6) | 6 ± 1.3
|     | (6) | (9) | (6) | 10.8 ± 1.5 | 12 ± 1.2 | 21.6 ± 2.5
|     | (6) | (9) | (6) | 1.36 ± 0.16 | 1.2 ± 0.4 | 2.2 ± 0.12
| 24  | 3.1 ± 0.3 | 4.6 ± 0.98
|     | (6) | (9) | (6) | 9 ± 0.53
|     | (6) | (9) | (6) | 20 ± 5 | 31 ± 2
|     | (6) | (9) | (6) | 36.4 ± 3.2
|     | (6) | (9) | (6) | 3 ± 0.23 | 3.4 ± 2.2 | 6.6 ± 0.56
| 48  | 10.8 ± 0.85 | 27 ± 0.9
|     | (6) | (9) | (6) | 15.5 ± 1.5
|     | (6) | (9) | (6) | 60 ± 3.2 | 150 ± 1
|     | (6) | (9) | (6) | 115 ± 8
|     | (6) | (9) | (6) | 5.5 ± 0.33 | 70 ± 5.5
|     | (6) | (9) | (6) | 7 ± 0.77
| 72  | 22.5 ± 2.5 | 68 ± 1
|     | (6) | (9) | (6) | 24 ± 1.3
|     | (6) | (9) | (6) | 103.0 ± 5 | 320 ± 6.5
|     | (6) | (9) | (6) | 200 ± 5.5
|     | (6) | (9) | (6) | 8.4 ± 0.74 | 45 ± 6
|     | (6) | (9) | (6) | 19 ± 0.28

Values are means ± SEM (n = 3), expressed as 10² pg/ml. *Significantly greater than non-treated: p < 0.01, bsignificantly greater than other irradiated value: p < 0.01.

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Table 4 Production of IL-8 stimulated by IL-1β

<table>
<thead>
<tr>
<th>Hrs</th>
<th>NP1</th>
<th></th>
<th></th>
<th>NP2</th>
<th></th>
<th></th>
<th>NP3</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-treated</td>
<td>10 Gy</td>
<td>20 Gy</td>
<td>Non-treated</td>
<td>10 Gy</td>
<td>20 Gy</td>
<td>Non-treated</td>
<td>10 Gy</td>
</tr>
<tr>
<td>6</td>
<td>27.6 ± 1</td>
<td>29 ± 0.42</td>
<td>30 ± 0.98</td>
<td>11.2 ± 0.56</td>
<td>110 ± 1.6a</td>
<td>16.6 ± 2.8a</td>
<td>3.2 ± 0.65</td>
<td>20 ± 1.4ab</td>
</tr>
<tr>
<td>24</td>
<td>216 ± 0.98</td>
<td>392 ± 2.2ab</td>
<td>208 ± 8.4</td>
<td>80 ± 0.6</td>
<td>500 ± 4.8ab</td>
<td>72 ± 2.8</td>
<td>19.2 ± 1</td>
<td>156 ± 9ab</td>
</tr>
<tr>
<td>48</td>
<td>272 ± 1.5</td>
<td>680 ± 4.25ab</td>
<td>340 ± 8.9a</td>
<td>104 ± 6</td>
<td>600 ± 9.8ab</td>
<td>92 ± 3.2</td>
<td>48 ± 0.85</td>
<td>192 ± 8.5ab</td>
</tr>
<tr>
<td>72</td>
<td>312 ± 2.2</td>
<td>720 ± 2.8ab</td>
<td>352 ± 7.9ab</td>
<td>132 ± 7.5</td>
<td>460 ± 8a</td>
<td>106 ± 2.8</td>
<td>48 ± 0.9</td>
<td>176 ± 9ab</td>
</tr>
</tbody>
</table>

Values are means ± SEM (n = 3), expressed as 10^3 pg/ml. aSignificantly greater than other irradiated value: p < 0.01.

II. Production of IL-8

Spontaneous production of IL-8 was about 100–10,000 pg/ml, gradually increasing to the maximum after 72 hours (Table 3). Production of IL-8 after stimulation by IL-1β was about 3000–500,000 pg/ml, and was maximum after 48 or 72 hours (Table 4). Spontaneous production increased more in the 20 Gy-irradiated cells than in the 10 Gy cells after 6 and 24 hours (p < 0.01). However, it increased more in the 10 Gy cells than in the 20 Gy cells after 48 and 72 hours (p < 0.01). Production stimulated by IL-1β increased more in the 10 Gy cells than in the 20 Gy cells (p < 0.01) after 24 hours.

Discussion

The cytokine IL-6 has multiple biological activities, some of which are involved in various aspects of immune and inflammatory responses. IL-6 controls the final maturation of B-cells into antibody-producing cells and stimulates immunoglobulin synthesis by plasmocytes. IL-6 can stimulate the activation of natural killer cells and the generation of cytotoxic T-lymphocytes. IL-6 acts as a hepatocyte-stimulating factor promoting the hepatic plasma protein synthesis of acute-phase proteins. IL-6 production is triggered by various extracellular stimuli in a number of different cell types including fibroblasts, macrophages, T- and B-lymphocytes, endothelial cells, keratinocytes and glial cells. IL-1, TNF and platelet-derived growth factor stimulate IL-6 production. IL-6 occurs in cells derived from several tumor types or cell lines including cardiac myxomas, uterine carcinomas, chronic lymphoblastic leukemias, bladder carcinomas and myelomas. In certain tumors such as cardiac myxomas, overproduction of IL-6 is presumed to be responsible for the generation of autoantibodies due to B-cell hyperactivation. IL-6 may also be an autocrine or paracrine growth factor for myelomas. In contrast, IL-6 can inhibit the growth of human breast and leukemia/lymphoma cell lines.

Expression of the IL-6 gene may have deleterious effects upon the host and therefore synthesis must be tightly controlled.

IL-8 is a cytokine produced by monocytes, fibroblasts and keratinocytes in response to stimulation by lipopolysaccharide, IL-1 or TNF-α. In vitro IL-8 stimulates chemotaxis in neutrophils, basophils and T-lymphocytes but not in eosinophils, and the release of lysosomal enzymes and superoxide anions from neutrophils. In vivo, IL-8 causes rapid neutrophilia following intraperitoneal injection in mice or intravenous injection in rabbits.

Glioma cells can release various cytokines such as an IL-1-like factor, an IL-3-like factor, IL-6, an IFN-β-like factor and transforming growth factor-β. However, the biological roles of these factors are unknown. This may imply that glioma cell lines constitutively express IL-6 and IL-8 genes. The effects of irradiation on cytokine production in glioma cells have not previously been investigated. Our results suggest that the production of IL-6 and IL-8 is enhanced by irradiation. However, the response to irradiation of IL-6 and IL-8 production did differ. IL-6 production was more enhanced by 20 Gy irradiation than 10 Gy irradiation, while IL-8 production was more enhanced by 10 Gy irradiation than 20 Gy irradiation.

The biological damage caused by x-rays is generally believed to be direct and indirect mediated by free radicals. Presumably irradiation acts on cytokine production at the transcriptional or translational level, etc. Akira et al. reported the isolation of a recombinant clone encoding NF-IL6, a nuclear factor involved in IL-6 gene expression. NF-IL6 bound to the transcriptional regulatory regions found in several acute-phase genes and other cytokine genes, including IL-8 and IL-1, implies that NF-IL6 may be involved in the regulation of acute-phase reaction, inflammation and hemopoiesis. However, other factors besides NF-IL6 may act in IL-6 and IL-8 production because of the differences in response to irradiation.
tion.

We always use radiotherapy for malignant glioma patients. The present study suggests that the cytokine network is changed by such irradiation, and the biological effect of irradiation should be considered from this approach.

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References

1) Akira S, Ishihiki H, Sugita T, Tanabe O, Kinoshita S, Nishio Y, Nakajima T, Hirano T, Kishimoto T: A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. EMBO J 9: 1897-1906, 1990

2) Bethea JR, Gillespie GY, Chung IY, Benveniste EN: Tumor necrosis factor production and receptor expression by a human malignant glioma cell line, D54-MG. J Neuroimmunol 30: 1-13, 1990


24) Raule FC, Shields J, Smith SH, Iliesei V, Merkenschlager M, Beverley PCL, Callard RE: B-
cell growth and differentiation induced by supernatants of transformed epithelial cell lines. *Eur J Immunol* 16: 1017-1019, 1986


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