Interleukin-1β induces apoptosis in GL15 glioblastoma-derived human cell line

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Interleukin-1β induces apoptosis in GL15 glioblastoma-derived human cell line. Am J Physiol Cell Physiol 279: C2043–C2049, 2000.—Interleukin 1-β (IL-1β) induces apoptosis in a glioblastoma-derived human cell line, exhibiting a poorly differentiated astrocytic phenotype. The apoptotic effect was demonstrated by analyzing nuclear morphology, in situ DNA fragmentation, and by ELISA detection of cytoplasmatic nucleosomes. We correlated the degree of differentiation of GL15 cells with the apoptotic response: 1) 4',6-diamidino-2-phenylindole staining, combined with glial fibrillary acidic protein (GFAP) immunofluorescence, showed that the cells with apototic nuclei express low levels of GFAP; and 2) at 13 days of subculture, in a more differentiated state, GL15 cells did not respond with apoptosis to IL-1β. In this cell line, nonrandom chromosome changes and the expression of SV40 early region have been previously shown. The involvement of p42/p44 mitogen-activated protein kinase (MAPK) pathway in the induction of apoptosis by IL-1β was hypothesized. Previous studies have shown that SV40 small T antigen partially inhibits phosphatase 2A, leading to an enhancement of the steady-state activity of p42/p44 MAPK pathway, PD-098059, specific inhibitor of p42/p44 MAPK pathway, counteracts the apoptotic effect of IL-1β, whereas SB-203580, specific inhibitor of p38 stress-activated protein kinase (SAPK) pathway, is ineffective. The imbalance between MAPK and SAPK pathways has been proposed as a key factor in determination of cell fate. Our results demonstrate that a further stimulation of p42/p44 MAPK pathway can constitute a death signal in tumor cells in which genomic damage and MAPK pathway control alterations occur.

CELL FATE IS REGULATED by integration of distinct and potentially conflicting signals. In different cellular systems, interleukin-1β (IL-1β) (8), a pleiotropic cytokine involved in regulation of immune and inflammatory responses, elicits such opposite responses as induction of proliferation or cell death. IL-1β is implicated in nervous system regeneration and reaction to injury (4, 22). In primary astrocytes, IL-1β induces proliferation, production of cytokines and lipid messengers, and nitric oxide synthesis (13, 15, 17, 30).

The effects of IL-1 on astrocytoma and glioma cell growth were investigated. Variable results were obtained: prevalent inhibitory but also stimulatory as well as no effects were observed. Recently, a cytostatic effect on several human glioblastoma cell lines was described (20).

We are studying the effects of IL-1β on GL15, a glioblastoma-derived human cell line (5, 6, 18) that exhibits, in normal conditions of culturing, a poorly differentiated astrocytic phenotype consisting of a mosaic expression (5–30% of cells) of glial fibrillary acidic protein (GFAP), marker of mature astrocytes (10, 11), and high expression of vimentin, the embryonic intermediate filament protein. The absence of serum and the presence of phorbol 12-myristate 13-acetate in the culture medium are able to induce a more differentiated phenotype (3) in which immunocytochemistry demonstrates an increase of the number of process-bearing cells labeled with GFAP antibody. Moreover, in long-term subculture, GL15 cells showed a time-dependent increase of the expression of mature astrocyte markers (18). Between 10 and 15 days of culture, GFAP levels dramatically increased, indicating a more

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differentiated phenotype in this condition. In this cell line, nonrandom chromosome changes have been shown, possibly related to the expression of the SV40 early region (26).

In recent years, a tight coupling of genomic damage to apoptosis has been established (12). In normal cells, DNA integrity is a prerequisite for normal cell cycle progression. The abnormal proliferation of tumor cells, in which genomic alterations occur, is allowed by the contemporary presence of antiapoptotic mutations and/or inhibition of tumor suppressor gene products.

GL15 cells, with a demonstrated contemporary presence of genomic damage along with the expression of the SV40 large T antigen, an inhibitor of tumor suppressor gene products, constitute a good experimental model to elucidate the molecular machinery underlying the peculiar responses of tumor cells to different stimuli.

MATERIALS AND METHODS

Cell cultures. Cultures of the GL15 glioblastoma multiforme cell line were grown in MEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU/ml penicillin G, 100 μg/ml streptomycin, and 1 mM sodium pyruvate. The flasks were incubated at 37°C in a 5% CO2 humidified atmosphere. The medium was changed twice weekly, and the cells were subcultivated when confluent.

GL15 cells were treated 3 days after trypsinization with 200 U/ml IL-1β (human, recombinant interleukin-1β, Boehringer Mannheim) in culture medium without serum at the indicated times. In some experiments, the treatment was performed in culture medium that contained 10% FBS. In some experiments, the concentration of IL-1β was between 1 and 200 U/ml. The other treatments were performed in the same conditions as those with IL-1β. In the experiments with okadaic acid, the final concentration of dimethyl sulfoxide (DMSO) was 0.25%. In the experiments with PD-098059 and SB-203580 (Calbiochem), the final concentration of DMSO was 0.2%. Controls were included at each experimental time point. When the combined action of each inhibitor with IL-1β was analyzed, this cytokine was added to the culture medium 90 min after the inhibitor.

Morphological analysis of the cells was performed with a contrast-phase Nikon microscope. Cell count was performed after trypsinization using a Burker camera.

Proliferation assay. [3H]Thymidine incorporation into the DNA was performed by incubating the cells with 5 μCi/ml [3H]thymidine (specific activity 24 Ci/mmol; Amersham) for 2 h, followed by washing with PBS and fixation with methanol. After three washes with 10% TCA, the pellet was solubilized with 0.5 N NaOH and 1% SDS and counted in a liquid scintillation spectrometer (Beckman).

Indirect immunofluorescence. Cells were extensively washed with phosphate-buffered saline (PBS), immersed in cold methanol, kept at −20°C for 7 min, and dried in air. The cells were then incubated for 60 min at room temperature with a rabbit anti-GFAP (Dakopatts, Denmark) polyclonal antibody (diluted 1:100 in PBS that contained 0.01% bovine serum albumin) and washed in PBS. After treatment with tetramethylrhodamine isothiocyanate-conjugated (Sigma) goat anti-rabbit IgG (diluted 1:100 in PBS that contained 0.01% albumin) and three washes with PBS that contained 0.1% Tween 20 and two washes with PBS, preparations were incubated with 2 μg/ml DAPI (4,6-diamidino-2-phenylindole; Sigma) for 5 min and dried in air. The same procedure was used with mouse anti-vimentin (Boehringer Mannheim) monoclonal antibody (diluted 1:4) and the anti-mouse IgG (Fab specific) fluorescein isothiocyanate-conjugated (Sigma) antibody (diluted 1:100), except for a permeabilization step performed by treatment with PBS that contained 0.1% Triton X-100 for 5 min. The preparations were observed with a DMRB Leika microscope.

In situ detection of DNA fragmentation. DNA fragmentation was detected by using the in situ cell death detection kit (Boehringer Mannheim), following the instructions of the manufacturer. After TdT-mediated dUTP nick-end labeling (TUNEL), fluorescein-labeled DNA strand breaks were analyzed under a fluorescence microscope.

ELISA detection of cytoplasmic nucleosomes. Determination of cytoplasmic histone-associated DNA fragments was performed by using the Cell Death Detection ELISA Plus Kit (Boehringer Mannheim), following the instructions of the manufacturer. The results are expressed as percentage of optical density, resulting from the activity of peroxidase-conjugated anti-DNA antibody complexed with cytoplasmic nucleosomes of treated cells, compared with the control.

SDS-PAGE and immunoblotting. GL15 cell cultures were washed with PBS and scraped with 62.5 mM Tris-HCl (pH 6.8), 2 mM EDTA, 0.5% Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride, and 0.1% SDS. The protein content was determined by the Bio-Rad protein assay kit.

The proteins were separated by SDS-PAGE in 10% acrylamide gel by the Laemmli method (16) and were then transferred to nitrocellulose filters according to Towbin et al. (27). Immunolabeling of GFAP and vimentin was performed as described by Tognon et al. (26). The primary antibody was a mouse anti-vimentin (Boehringer Mannheim) monoclonal antibody. The secondary antibody used to detect vimentin content was the peroxidase-conjugated rabbit immunoglobulins to mouse immunoglobulins (Dakopatts).

NADPH-diaphorase cytochemistry. After fixation with 4% paraformaldehyde in PBS (pH 7.4), the cells were rinsed in PBS. The cells were incubated in a solution composed of 1 mM NADPH (Sigma), 0.6 mM nitro blue tetrazolium (Sigma), and 0.2% Triton X-100 in 100 mM Tris·HCl (pH 7.5) for 40 min at 37°C (28). Samples incubated in the stain medium devoid of NADPH constituted the control for the reaction specificity.

Fig. 1. Effects of interleukin-1β (IL-1β; 200 U/ml) on GL15 cells 4 days after treatment. A: contrast-phase microscopy, control. B: contrast-phase microscopy, IL-1β (200 U/ml). C: immunofluorescence staining of glial fibrillary acidic protein (GFAP), control. D: 4′,6-diamidino-2-phenylindole (DAPI) staining, control (same field as in C). E: immunofluorescence staining of GFAP + IL-1β (200 U/ml). F: DAPI staining + IL-1β (200 U/ml) (same field as in E). G: immunofluorescence staining of vimentin, control. H: DAPI staining, control (same field as in G). I: immunofluorescence staining of vimentin + IL-1β (200 U/ml). J: DAPI staining + IL-1β (200 U/ml) (same field as in J). K: in situ detection of DNA fragmentation by TdT-mediated dUTP nick-end labeling (TUNEL) method, control. L: DAPI staining, control (same field as in K). M: in situ detection of DNA fragmentation by TUNEL method + IL-1β (200 U/ml). N: DAPI staining + IL-1β (200 U/ml) (same field as in M). Arrows in M and N indicate DNA fragmentation in apoptotic nuclei. Original magnification, ×400.
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RESULTS

In GL15 cells, IL-1β (200 U/ml) induced, 2 days after treatment, an evident morphological change: several cells became rounded and were less adherent to the substrate, and some giant cells began to appear.

Four days after treatment, the number of giant cells increased, as well as the number of less adherent cells; several cells were completely detached (Fig. 1, A and B). The cell count showed that the number of cells did not vary significantly until the fourth day after treatment, when the number of cells was 70% ± 9% (SD) compared with the control. The decrease in cell number, induced by the treatment, performed in the absence of serum and at cell semiconfluence, mostly monitored the detachment of the cells from the substrate.

Analysis of [3H]thymidine incorporation into DNA shows that, at 3 and 4 days after treatment, there was a significant decrease of DNA synthesis induced by IL-1β, compared with the control (Fig. 2).

DAPI staining of nuclei showed that, at 4 days, treatment with IL-1β increased the fraction of apoptotic nuclei from 2.3% ± 0.7% (SD) to 16.2% ± 1.3% (SD) (P < 0.001). Morphology of apoptotic nuclei was characterized by condensed chromatin or nuclei (Fig. 1, F, J, and N). Similar results were obtained when concentrations of IL-1β between 200 U/ml and 1 U/ml were used, thus excluding the possibility of a biphasic response of GL15 cells to IL-1β, with a stimulating effect of this cytokine at lower doses and an apoptotic effect at higher doses.

In situ detection of DNA fragmentation by the TUNEL method (Fig. 1, K and M) shows that the apoptotic structures with condensed chromatin are heavily labeled, whereas fused nuclei do not seem stained by fluorescein.

A quantitative analysis of DNA fragmentation was performed by an ELISA method, detecting the amount of cytoplasmic nucleosomes (see MATERIALS AND METHODS). At 2 and 4 days after treatment, IL-1β induced a significant increase of cytoplasmic nucleosomes; this result is a further demonstration of the apoptotic effect of IL-1β on GL15 cells (Fig. 3A). Similar results were obtained when the treatment with IL-1β was performed in the presence of 10% serum in the culture medium (Fig. 3B).

Because of their neoplastic origin, we tried to correlate the degree of differentiation of GL15 cells with the apoptotic response. In our experimental conditions, the GL15 cell line showed a mosaic expression of GFAP, marker of mature astrocytes. DAPI staining, combined with GFAP immunofluorescence, demonstrated that the cells with apoptotic nuclei expressed low levels of GFAP (Fig. 1, C–F). The same technique applied to evaluate the expression of vimentin, the embryonic intermediate filament protein, showed that vimentin, on the other hand, is expressed in apoptotic cells (Fig. 1, G–J). This result indicated that the low level of GFAP in apoptotic cells was not due to a general destruction of cytoskeleton.
Western blot analysis showed that IL-1β at 24 h of treatment induced a selective degradation of GFAP [60% ± 15% (SD) compared with the control] (Fig. 4B), while vimentin was not affected (Fig. 4A).

In long-term subculture, GL15 cells exhibited a spontaneous differentiation in vitro, with a strong increase of GFAP levels between 10 and 15 days. We investigated the effects of IL-1β on GL15 cells in this more differentiated state. Our results demonstrate that IL-1β is unable to induce apoptosis on GL15 cells when treated at 13 days of subculture (Fig. 3C). Our findings demonstrate a peculiar response of GL15 cells to IL-1β. On the other hand, IL-1β elicits a classic astrocytic response in GL15 cells: an increase of diaphorase activity (Fig. 5, A and B).

The selective induction of apoptosis in glioma cells, exhibiting differentiation impairment, could be, in our opinion, a good model for the study of the possible mechanisms involved.

The expression of the SV40 large T antigen, and then of the small T antigen, in the GL15 cell line prompted us to hypothesize an involvement of the mitogen-activated protein kinase (MAPK) pathway (21, 24) in the induction of apoptosis by IL-1β. Partial inhibition of phosphatase 2A by small T antigen maintained the MAPK pathway in an activated state (2, 25). A further stimulation of this pathway could constitute an unbalanced induction of the proliferation machinery in cells carrying karyotypic abnormalities.

Okadaic acid, a phosphatase inhibitor, is currently being used to study the relationship between mitosis and apoptosis (19). At very low concentrations, it could be considered a specific inhibitor of phosphatase 2A. The treatment of GL15 cells with 10 nM okadaic acid, in the same conditions as those used for IL-1β treatment, at 3 days, induces apoptosis as well as an indiffentiation effect (Fig. 5, C and D). However, this result is not a direct demonstration of the involvement of the p42/p44 MAPK pathway in the apoptotic response of GL15 cells to IL-1β.

A more direct demonstration of the involvement of the MAPK pathway on induction of apoptosis by IL-1β was obtained by using a very specific MAPK kinase inhibitor, PD-098059 (1, 9), and a very specific inhibitor of p38 stress-activated protein kinase (SAPK), SB-203580 (7, 23). Two days after treatment, PD-098059 counteracted the apoptotic effects of IL-1β (Fig. 5, E, F, and H), whereas SB-203580 was ineffective (Fig. 5J). DAPI staining of nuclei showed that, 2 days after treatment, IL-1β increased the number of apoptotic nuclei from 3% ± 0.9% (SD) to 11% ± 4.4% (SD). After treatment of GL15 cells with IL-1β in the presence of PD-098059, the percentage of apoptotic nuclei was 3.2% ± 1% (SD), whereas in the presence of SB-203580 it was 9% ± 0.7% (SD). The comparison between the percentage of apoptotic nuclei after treatment with IL-1β alone [11% ± 4.4% (SD)] and after treatment with IL-1β and PD-098059 [3.2% ± 1% (SD)] indicated a significant (P < 0.001) decrease in apoptotic nuclei induced by the presence of PD-098059. This result indicated the involvement of a MAPK pathway on induction of cell death by IL-1β. At 4 days, apoptosis was observed in cells treated with IL-1β and PD-098059. The mechanism underlying this phenomenon requires further investigation.

**DISCUSSION**

The concept that cell death, proliferation, and differentiation partially share common control mechanisms is beginning to be supported by some experimental evidence. Our results show that IL-1β induced apoptosis on a glioblastoma-derived human cell line, exhibiting a poorly differentiated astrocytic phenotype.

In an attempt to correlate the degree of differentiation of GL15 cells with the apoptotic effect of IL-1β, we used the combined techniques of GFAP immunofluorescence and DAPI staining. Apoptotic cells showed a low level of GFAP expression. These results could indicate a general degradation of cytoskeleton; but, vimentin, evaluated with the same technique, was still present in apoptotic cells. Analysis of GFAP and vimentin content by immunoblotting confirmed this result: IL-1β, 1 day after treatment, induced a significant decrease of GFAP, whereas vimentin was not affected.

In long-term subculture, GL15 cells exhibited a spontaneous differentiation in vitro, with a strong increase of GFAP levels between 10 and 15 days. In this
more differentiated state, GL15 cells did not respond with apoptosis to IL-1β. This result constitutes a further demonstration that the cells exhibiting a less differentiated phenotype are more sensitive to the apoptotic effect of IL-1β.

A cytostatic effect of IL-1β on some human glioblastoma cell lines has been recently shown (20). The peculiar apoptotic response of GL15 cells could be due to the imbalance of MAPK pathways. In these tumor cells, where genomic alterations have been demon-
strated, survival and proliferation capabilities were possibly sustained by the expression of the SV40 early genes. The presence of the SV40 small T antigen partially inhibited phosphatase 2A, leading to an enhancement of the steady-state activity of the p42/p44 MAPK pathway.

The functional distinction between MAPK and SAPK pathways, inside the MAPK superfamily, derives from the findings that MAPKs are preferentially activated by polypeptidic growth factors and tumor-promoting phorbol esters, whereas SAPKs are generally activated by stress stimuli and proinflammatory cytokines. In several cellular systems, the activation of SAPK pathways are involved in the initiation of apoptosis or, alternatively, in a cytostatic effect, while proliferation and differentiation are supported by activation of the p42/p44 MAPK pathway. The balance between these intracellular systems has been proposed as a key factor in determination of cell fate (14, 29).

Our results demonstrate that an inhibitor of the p42/p44 MAPK pathway counteracts the apoptotic effect of IL-1β on GL15 cells. The partial inhibition of phosphatase 2A by the SV40 small T antigen maintains the p42/p44 MAPK pathway in an activated state. Our results demonstrate that a further stimulation of this pathway can constitute a death message in tumor cells, in which genomic damage and MAPK pathway control alterations occur.

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