Vol. 205, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1994 Pages 215-220

SELECTIVE CYTOTOXICITY OF TRANSFORMED CELLS BUT NOT NORMAL CELLS BY A SIALOGLYCOPEPTIDE GROWTH REGULATOR IN THE PRESENCE OF TUMOR NECROSIS FACTOR [1,2]

Keith M. Woods, Heideh Fattaey, Terry C. Johnson, and Stephen K. Chapes*

Division of Biology, Kansas State University
Manhattan, Kansas 66506

Received September 28, 1994

SUMMARY: The tumor necrosis factor-α (TNF)-resistant, SV40-transformed, murine fibroblast cell lines, F5b and F5m, became sensitive to TNF-mediated cytolysis after treatment with a biologically active 18 kDa peptide fragment (SGP) derived from a 66-kDa parental cell surface sialoglycoprotein. Neither TNF nor the SGP alone exhibited cytotoxicity to the two SV40-transformed cell lines. However, Balb/c 3T3 cells, incubated with SGP alone or with SGP and TNF, were not killed. Therefore, SGP can selectively sensitize cells for TNFα–mediated cytotoxicity. This selective sensitization may be due to the previously documented ability of the SGP to selectively mediate cell cycle arrest.

The cells used in this study, 3T3, F5b and F5m, are resistant to killing by TNF (1,2). However, TNF sensitivity can be induced in these cells by inhibiting cellular protein synthesis (2) and it has been suggested that the synthesis of specific protein(s) may be required for the establishment of resistance to soluble TNF (3). TNF plays an important role in immune responses against virus and intracellular bacterial infections by destroying the infected cells (2,4,5). Perhaps infected cells are targeted for destruction by TNF because of an alteration in cellular metabolism as a result of the infection. Some tumor cells can be made susceptible to TNF cytolysis. For example, F5b cells, normally resistant to TNF, are susceptible to killing by activated macrophages in a contact-dependent

[1] Kansas State Agricultural Experimental Station Publication # 95-129-J.

[2] This work was supported in part by U. S. Army Research and Development Command grant # DAMD 17-89-Z-9039, American Heart Association grant KS-94-GS-3, NASA grants NAGW-1197 and NAGW-2328 and the Kansas Health Foundation.

* Corresponding author. Fax: (913) 532-6653.

Abbreviations: SGP, sialoglycopeptide; TNF, tumor necrosis factor-alpha.

0006-291X/94 $5.00

manner involving TNF (2). These data suggest that tumoricidal macrophages may induce TNF sensitivity in tumor cells by inhibiting or altering the metabolism of the target cells. Our work with macrophages led us to query whether a physiologically relevant mechanism existed to sensitize cells to TNF. We found that a purified 18 kDa peptide fragment (SGP), proteolytically generated from a 66 kDa membrane sialogycoprotein, expressed on bovine cortex cells inhibited cellular metabolic processes (6). This naturally occuring cell surface ligand is a biologically relevant inhibitor of DNA synthesis, protein synthesis and cellular proliferation (6, 7, 8). The inhibitory activity of SGP occurs by binding to a specific cell surface receptor (9, 10). Moreover, the SGP inhibits an unusually wide array of cells, at nanomolar concentrations (7).

Given the inhibitory nature of the SGP, we tested whether this naturally occurring cell cycle regulator could sensitize cells to cytolysis by TNF. Although not cytotoxic by itself under these experimental conditions, we found that SGP did selectively sensitized the TNF-resistant transformed cell lines, but not nontumorigenic 3T3 cells, to the cytotoxic action of TNF .

Materials and Methods

Cell lines The SV40-transformed murine fibroblast cell lines, F5b, which is sensitive to macrophage-mediated killing, and F5m, the macrophage-resistant sister clone of F5b, have been described previously (1,11,12). The nontransformed cell line Balb/c 3T3 (3T3) was obtained from the American Type Culture Collection. The cells were maintained in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) supplemented with 10% Opti-MEM (Gibco), 2% fetal bovine serum and 0.3% L-glutamine (Gibco). No antibiotics or antimycotics were used in the continuous culture of these cells.

TNF assay 1 x 10^4 ^{51}Cr-labelled target cells were added to each well of a 96-well microtiter plate and 50 or 100 nM (final concentration) of the SGP, diluted in phosphate buffered saline (PBS, pH 7.2),were added. Controls included target cells in medium alone, or a medium containing 32 Units/ml rTNF (20 pg/unit, Genzyme, Cambridge, MA), or a medium containing 30 Units/ml SGP (500 ng/unit). The assay was incubated for 16 hours at 37^{o}C in 8% CO_2. Ninety µl of the supernatant was removed to be counted for Cr release and the percent cell death (%CD) was calculated according to the following equation:

$$\%CD = \frac{\text{(Experimental Cr release - control spontaneous Cr release)}}{\text{(Maximal Cr release - Control spontaneousl Cr release)}} \times 100$$

Using the same cells cell death was determined using MTT. To the remaining medium, 50 µl of 2 mg/ml (1-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT, Sigma, St. Louis, MO) in normal saline were added to each well and incubated for an additional 3 hrs. The medium was then aspirated and the formazan crystals dissolved in 50 µl of isoPBS (80% isopropanol, 20% phosphate buffered saline (PBS) with 40 µl 5N HCl/100 ml). Absorbance was read at 570 nm and % cell death (%CD) calculated from the following equation:

$$\%CD = \frac{\text{(Experimental Abs - Medium Control Abs)}}{\text{(0.001 - Medium Control Abs)}} \times 100$$

Isolation and purification of SGP The bovine SGP was purified as previously described (6). Briefly, SGP was isolated from intact bovine cerebral cortex cells by mild proteolysis, ethanol precipitated and extracted with chloroform/methanol (2:1 v/v). After overnight dialysis against H_2O, the SGP was lyophilized, then purified by DEAE ion-exchange chromatography, followed by HPLC using a TSK3000 column (Phenomenex, Rancho Palos Verde, CA). A unit of activity is defined as the amount of peptide required to inhibit protein synthesis 25 % in 30 min. at 37° C (specific activity varies between purification).

Protein synthesis assay Cells were transferred to 2 ml of medium (MEM-Hepes; Hazelton, 0.6% Hepes, U.S. Biologicals) and were centrifuged for 2-3 minutes at 300 x g. The cells were then resuspended to a concentration of 4 x 10^6 cells/ml in methionine-free medium. Forty µl of each SGP dilution was put into a 13 x 100 mm disposable tube and 100 µl of the cell suspension was added. The tubes were then covered with parafilm and incubated at 37°C for 30 minutes while shaking. 10 µl of [^{35}S]-methionine, diluted in methionine-free medium (2 µCi/ml), was added to the tubes containing the cells and was incubated for an additional 15 minutes. 2 ml of cold PBS was then added and the cells were centrifuged at 700 x g for 3 minutes. The supernatant fluid was discarded and the pellet was resuspended in 100 µl of deionized H_2O and vortexed. 100 µl of 1N NaOH was added to dissolve the cells and vortexed. To this, 600 µl of 20% trichloroacetic acid (TCA) was added. Precipitated protein was pelleted by centrifuging at 700 x g for 15 minutes. The supernatant fluid was discarded and the pellet was washed two additional times. [^{35}S]-methionine incorporation was determined with a scintillation counter.

Results and Discussion

The versatility of TNF as a cytolytic molecule is illustrated in Table 1. The TNF-resistant cells, F5b, F5m and 3T3, became sensitive to TNF in the presence of the protein synthesis inhibitor, cycloheximide (CHX). A similar inhibitory response, with respect to protein synthesis, was seen in tumor cells and nontumor cells by SGP as determined by

Table 1. Effects of Cycloheximide on Resistance to TNF of 3T3, F5b and F5M Cells

Target[a]	32 U/ml TNF Treatment[b]	% Cell Death[c]
3T3	None	0 ± 0
	10 µg/ml CHX	83 ± 1
F5b	None	0 ± 0
	10 µg/ml CHX	66 ± 2
F5M	None	0 ± 0
	10 µg/ml CHX	47 ± 1

a One x 10^4 cells were seeded per well of a microtiter plate.
b 32 U/ml rTNF (20 pg/U). Cycloheximide (CHX).
c Cell death determined by MTT. Values represent mean ± SD of triplicate samples.

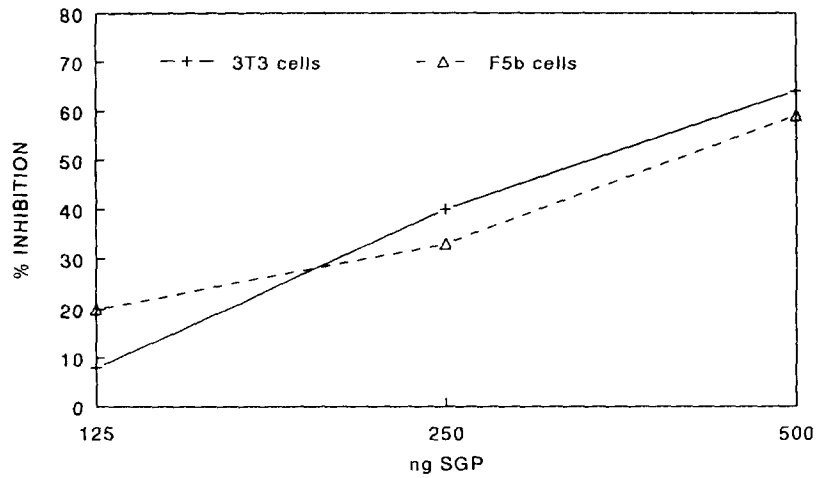

Figure 1. Dose response of SGP inhibition of protein synthesis of F5b and 3T3 cells. 2-3 x 10^5 Cells were incubated in 140 µl medium for 30 min containing 125, 250 and 500 ng SGP (final concentrations 50, 100, and 200nM, respectively). Protein synthesis was determined by [^{35}S] methionine incorporation.

[^{35}S]-methionine incorporation. At concentrations of 125, 250 and 500 ng/ml of the SGP, protein synthesis of both normal and transformed cells showed similar inhibition (Figure 1). The ability of these cells to respond to SGP indicates that these cells expressed the appropriate receptor for the inhibitor.

Because SGP and CHX inhibited protein synthesis in 3T3 and F5b cells similarly, we determined whether SGP would also induce sensitivity to TNF-mediated cytolysis. Cells were incubated with 32 U/ml recombinant TNF (rTNF) in the presence of a concentration of the SGP known to inhibit cell proliferation (Figure 2). SGP selectively sensitized F5b cells to TNF-mediated cytolysis but did not affect 3T3 cells (Figure 2). We

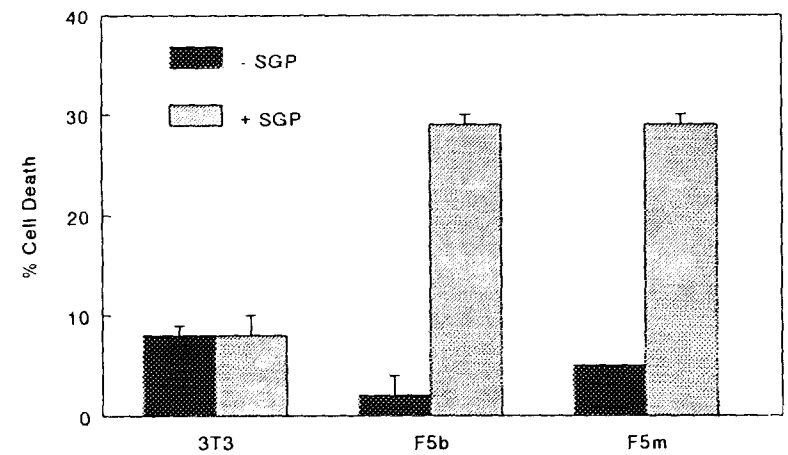

Figure 2. F5b, F5m and 3T3 cells were incubated with 3 units of SGP/well in the presence of 32 units/ml rTNF (final concentration 416 nM SGP, 12.5 pM rTNF). Results determined after 16 hrs by specific ^{51}Cr release.

also examined whether F5m cells, a sister clone to F5b, could be sensitized to TNF by SGP and found that they also were sensitized to TNF-mediated cytolysis as determined by a standard ^{51}Cr release assay (Figure 2). This was also determined using MTT to quantitate cell death (data not shown). Furthermore, the cell surface-derived SGP, exhibited a selectivity in sensitizing tumor cells to TNF-mediated killing similar to tumoricidal macrophages (2).

Unlike synthetic inhibitors of protein synthesis, SGP only induced the SV40-transformed tumor cells to be lysed by TNF while SGP alone was nontoxic. The growth regulatory properties of SGP probably were responsible for the discrimination since protein synthesis inhibtion of 3T3 cells and the virus-transformed cells was similar (Figure 1). Previous studies found that SGP arrested 3T3 cells in the G_1 phase of the cell cycle (7). In contrast, Enebo *et al.* (13) showed that F5b cells were relatively refractory to proliferation inhibition by the SGP. The SGP-induced TNF sensitivity of F5m and F5b cells might have occurred because of their inability to become efficiently arrested in the G_1 phase of the cell cycle; whereas 3T3 cells were arrested and thereby protected from cytolysis. This is an attractive hypothesis because TNF-mediated cytolysis has been linked to the cell cycle at G_1/S phase boundary (14). Furthermore, Van der Bosch *et al.* reported that the resistance of target cells to cytotoxicity was dependent on growth arrest in the G_1 phase of the cell cycle (15). This hypothesis is also consistent with observations that cell cycle arrest by the SGP was regulated by the retinoblastoma gene product (RB) by phosphorylation events (16) and that cells transformed by DNA tumor virus or viral oncogene products (e.g. adenovirus E1A and papilloma virus E6/E7) also were not responsive to cell cycle arrest by the SGP (13). The process of cellular transformation by the simian virus-40 (e.g. F5b and F5m cells,) involves the inactivation of the regulatory suppressor functions of the RB protein through complexes formed with the large T antigen (16).

By acting as a cell cycle regulator the SGP (7,13) presumably induces TNF-mediated lysis in a cell cycle specific manner which is consistent with the way human monocytes killed tumor cells (15). Both macrophages and SGP appear to select tumor cells for killing and to spare normal cells. This is relevant because macrophages kill F5b cells in a contact-dependent process using TNF (2). Although we have been unable to determine whether SGP is involved in macrophage-mediated killing due to the lack of a neutralizing antiserum against SGP, the inhibitor may be a useful tool to investigate the mechanism of action of TNF without resorting to artificial and somewhat nonspecific means of sensitization (e.g. inhibitors of mRNA and protein synthesis).

References

1. Chapes,S.K., Duffin,D. and Paulsen, A. (1988) J. Immunol. 140, 589-596.

2. Woods, K.M. and S.K. Chapes. (1993) J. Leuk. Biol. 53, 37-44.

3. Kirstein, M. and Baglioni, C. (1986) J. Biol. Chem. 261, 9565-9567.

4. LeBlanc, P.A., Heath, L.S. and Um, H-D. (1990) J. Leuk. Biol. 48, 1-6.

5. Klimpel, G.R., Shaban, R. and Niesel, D.W (1990) J. Immunol. 145, 711-717.

6. Sharifi, B.G., Johnson, T.C., Khurana, V.K., Bascom, C.C. and Chou, H-H. (1986) J. Neurochem. 46, 461-469.

7. Fattaey, H.K., Johnson, T.C. & Chou, H-H. (1989) J. Cell. Physiol. 139, 269-274.

8. Fattaey, H.K., D.J. Enebo, P.J. Moos and Johnson,T.C. (1993) J. Cell. Biochem. 52, 69-77.

9. Bascom, C.C., Sharifi, B.G. and Johnson, T.C. (1986) J. Cell Physiol. 128, 202-208.

10. Sharifi, B.G. and Johnson, T.C. (1987) J. Biol. Chem. 262, 15752-15755.

11. Chapes, S.K. and Gooding, L.R. (1985) J. Immunol. 135, 2192-2198.

12. Rosenkrans, Jr.,C.F. and Chapes, S.K. (1991) Cell. Immunol. 133, 187-193.

13. Enebo, D., Fattaey, H.K., Moos,P.J. and Johnson, T.C. (1994) J. Cell. Biochem. 55, 200-208.

14. Coffman, F.D., Haviland, D.L.,Green, L.M. and Ware,C.F. (1989) Growth Factors 1, 357-364.

15. Van der Bosch, J., Rueller, S., Horn, D., Schumann, R., and Schlaak, M. (1990) Exp. Cell Res. 187, 185-192.

16. Ludlow, J.W., DeCaprio, J.A., Huang, C-M., Lee, W-H., Paucha, E., and Livingston, D.M. (1989) Cell 56, 57-65.