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SHORT COMMUNICATION

Cyclins D1, E, A Expression and Synergistic Cytotoxicity to a Cholangiocarcinoma Cell Line from Recombinant TNF-α and PHA Supernate

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SUMMARY **We studied the cytotoxic effects of recombinant TNF-α and supernate of phytohemagglutinin stimulated peripheral blood mononuclear cells individually and in combination against a cholangiocarcinoma cell line. Levels of cyclins D1, E and A in the cell line were detected by immunoblotting, and the cell cycle stage was assayed by propidium iodide staining followed by flow cytometry analysis. Viable and apoptotic cells were assessed by trypan blue dye exclusion, DAPI staining, agarose DNA laddering and propidium iodide staining. At the beginning of each experiment, the majority of cholangiocarcinoma cells expressed cyclin A and were in S phase as determined by propidium iodide staining. Treatment of such cells with recombinant TNF-α resulted in cytotoxic effects clearly evident at 36 hours post exposure. There was a synergistic killing effect when recombinant TNF-α was combined with PHA supernate and this effect could be partly neutralized by monoclonal anti TNF-α, interleukin (IL)-2, IL-12 and IFN-γ.**

One reason for failure of the immune response against tumor cells is the evasion of both humoral and cell mediated immune mechanisms.[1-3] To tackle this problem, several studies have been carried out and a variety of experimental and clinical approaches have been developed for immunotherapy using immune cells or the immune soluble mediators, cytokines.[4-7] For example, the uses of recombinant cytokines such as TNF-α, IFN-α, IFN-β, IFN-γ, interleukin (IL)-1, IL-2, IL-4, IL-5, IL-12, have been reported either individually or in combination. However, the most notable obstacle to this approach is unwanted side effects and the delicacy of the cytokine networks.[8-9]

The immune response against tumors is a complex interaction between individual host factors and individual tumor factors. To study the roles of both players, we have chosen a cholangiocarcinoma cell line model with cyclins D1, E and A expression and the cell cycle stage as markers of tumor response to host soluble immune mediators. Cytotoxic activities of the soluble mediators, i.e. recombinant TNF-α and supernate from PHA stimulated peripheral blood mononuclear cells, were tested singly and in combination against this tumor cell line. TNF-α was found to be cytotoxic to the cholangiocarcinoma cells at S phase when used singly or in synergistic combination with the supernate.

MATERIALS AND METHODS

The cholangiocarcinoma cell line used was a gift from Prof. Stitaya Sirisinha, Mahidol University.[10] It was maintained in tissue culture flasks using RPMI-1640 culture medium supplemented with 10% fetal bovine serum (FBS) at 37°C. To obtain the majority of cells at the same cell cycle stage for use in the experiments, cells at confluency were subjected to serum deprivation for 24 hours by replacement of normal growth medium with RPMI-1640 lacking FBS. This

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was followed by restoration of 10% FBS RPMI-1640 for 24 hours (or 48 hours from beginning of serum deprivation) at the start of each experiment. Preliminary tests detecting cell cycle phase and cyclins D1, E, A expression showed that the GO/G1 to S transition in these cultures occurred at 48 hours, so immune mediators were added at a cell age of 48 hours and the effects tested at 36, 48 and 72 hours thereafter.

To obtain soluble mediators in the supernate of phytohemagglutinin (PHA) stimulated peripheral blood mononuclear cells, blood samples from 5 females and 4 males age 20-30 years were stimulated with 2 μg/ml PHA (Sigma Chemical Co., St. Louis, USA) for 48 hours at 37°C and then pooled and kept frozen as "PHA supernate" until use. Recombinant human TNF-α (rTNF-α) was purchased from Sigma Chemical Co.

For cytotoxicity assays, triplicate cultures of cholangiocarcinoma cells maintained as described above were exposed to 30 μg/ml rTNF-α in the presence of 0.75 μg/ml actinomycin D and 200 μl "PHA supernate" singly or in combination at 37°C, in a 5% CO_2 humidified incubator. Control cultures were treated in a similar man-ner except for the addition of carrier solutions. Samples were as-sayed at 36, 48 or 72 hours of incubation. Cell death was quantified using trypan blue dye exclusion with a light microscope and DAPI (Boehringer Mannheim, Germany) staining with a fluorescence microscope. Briefly, washed cells were stained with DAPI solution for 15 minutes and destained with methanol for 15 minutes and mounted with 50% glycerine and then visualized under fluorescence microscope (excitation at 372 nm and emission at 454 nm).

The cell cycle phase of treated and control cholangiocarcinoma cell cultures was determined by flow cytometry analysis using propridium iodide staining (Cycle test™ plus DNA Reagent kit, Becton Dickinson). The cellular DNA content was analyzed by flow cytometry. Expression of cyclins D1, E, A was determined by immunoblot staining with anti-cyclin D1 (pharMingen), anti-cyclin E (pharMingen) and anti-cyclin A (Serotec) monoclonal antibodies. Briefly, cell protein extracts were prepared by ultrasonic disruption using a sonicator, protein concentrations were quantified by Lowry method adjusted to 30 μg/ml after which a 12% SDS polyacrymide gel electrophoresis was done, and blotted onto nitrocellulose membrane with a pore size of 0.45 μm (Pharmacia Biotech). The monoclonal antibodies were stained and visualized for the positive bands by alkaline phosphatase staining.

The agarose DNA ladder assay of DNA extracted from cells with RNase I and proteinase treatment was done concurrently with the propidium iodide staining for flow cytometry analysis. Briefly, DNA extract was horizontally electrophoresed in 1.8% agarose gel with 0.1 μg/ml ethidium bromide with 100 bp DNA marker and visualized under an UV lamp.

The neutralization of cytotoxic effects due to TNF-α and the synergistic effect of "PHA supernate" was accomplished by the addition of the monoclonal antibodies to the cell cultures as fol-lows: anti-TNF-α (Sigma) 1.0 μg/ ml, anti-IL-2 (Sigma) 6.0 μg/ml, anti-IL-12 (Sigma) 4.0 μg/ml and anti-IFN-γ (Sigma) 0.1 μg/ml (concentrations of each monoclonal antibody were obtained from a preliminary test) together with TNF-α and "PHA supernate" as described for the assays above.

RESULTS AND DISCUSSION

Staining immunoblots of extracts from the control culture of cholangiocarcinoma cells showed a positive expression of cyclin A in the experimental assay at the 48 hour cells or cells at 0 hour after treatment, and at the 36 hour cells without treatment (Table 1). By propidium iodide staining and flow cytometry analysis, this corresponded to the cell culture shift from 13% of cells in M2 (S) before serum deprivation to 35% of cells at 48 hours or 0 hour after treatment (Table 1).

Treatment with rTNF-α or rTNF-α in combination with "PHA supernate" did not change cyclin D1 expression (Table 1). However, cyclin E expression was induced by rTNF-α treatment only. The latter phenomenon suggested that cells were leaving M3 (G2/M) to M4 (dead cell) (Table 1). The majority of living cell (40-60%) remained in M1 after treatment with rTNF-α or with PHA supernate. In contrast to rTNF-α or "PHA supernate" treatment alone, combination treatment did not induce cyclin E expression, moreover the majority of dead cells (70-80 %) was detected in M4 (Table 1).

The preliminary study of the cytotoxic susceptibility according to tumor cell age for treatment with rTNF-α or rTNF-α combined with "PHA supernate" elucidated a decrease of viable cells after treatment when using tumor cell age of 48 or 72 hours. It was dem-

Table 1 Cell cycle distribution by flow cytometry analysis and cyclins D1, E, A detection by immunoblot staining of cells

Condition of treatment	Cell (%)				Cyclin expression		
	M1 (G0/G1)	M2 (S)	M3 (G2I/M)	M4 (dead cells)	D1	E	A
48 h before treatment	78	13	9	0	nd	nd	nd
0 h treatment	44	36	10	7	+	-	++
36 h TNF-α treatment	58	11	4	26	+	++	+
36 h PHA supernate treatment	44	21	5	22	nd	nd	nd
36 h TNF-α + "PHA supernate" treatment	16	5	2	76	±	-	+
36 h without treatment	55	32	5	4	±	-	++

nd = not done

Table 2 Neutralizing effect of anti-TNF-α, IL-2, IL-12 and IFNγ and trypan blue dye exclusion and flowcytometry analysis

Treatment	Concentration (μg/ml)	% Dead cell	
		Trypan blue exclusion	Flow cytometry analysis
Anti -TNF-α	1.0	55	56
Anti-IL-2	6.0	79	73
Anti-IL-12	4.0	60	51
Anti-IFNγ	0.1	56	64
Control (TNF-α + PHA supernate)	None	84	87

onstrated that the highest cytotoxic susceptibility to the treatment of rTNF-α or rTNF-α combined with "PHA supernate" was at 36 hours, as observed by trypan blue staining, DAPI staining and DNA laddering (data was not shown). Neutralization of anti TNF-α, IL-2, IL-12 and IFN-γ showed a rebound effect on cytotoxicity (Table 2) that was comparable to the increased amount of cell debris by flow cytometry analysis.

The results of our study showed that cytotoxic susceptibility of tumor cells to rTNF-α alone or in combination with "PHA supernate" tended to be tumor cell stage sensitive. The synergistic cytotoxic effect of "PHA supernate" when combined with rTNF-α suggested that it may influence the cell cycle machinery. The study by Volland *et al.*[11] showed an accelerating role for TNF on tumor growth under low glucose supply. Our study suggested that the "PHA supernate" could drive cells to enter the S phase of the cell cycle and this coincidentally indicated the non-toxic effect of PHA supernate. When tumor cells entered the S phase, the soluble mediators in "PHA supernate" combined with rTNF-α or rTNF-α alone can trigger programmed cell death. The neutralizing effect of anti IL-2, IL-12 and IFN-γ on the cytotoxic activities of "PHA supernate" in combination with rTNF-α can be explained by the interplay of various cytokines, as shown in many systems.[12-15] Moreover, it indicated that the soluble immune mediators in our "PHA supernate" might play a role in cholangiocarcinoma treatment by driving cells to the S phase of the

cell cycle where TNF-α can induce cell death effectively.

Interaction of tumor cells and various soluble immune factors may give different outcomes. For example, cholangiocarcinoma cells may be more cell phase dependent with regard to cytotoxic susceptibility than other tumor cells.[16] The synergistic cytotoxic activity of our rTNF-α and "PHA supernate" in combination and the rebound effect by anti-IL-2, IL-12 and INF-γ illustrate the different effects of soluble mediators. Our hypothetical mechanism for the synergistic cytotoxic activity is that IL-2, IL-12 or IFN-γ may function by driving cells into the S stage of the cell cycle or by priming cells for high sensitivity to the death mechanism induced by TNF-α.

Although the soluble mediators in our "PHA supernate" are not classified, the results of the neutralizing assay indirectly suggest the presence of IL-2, IL-12 and IFN-γ. IL-2, IL-12 or IFN-γ alone or in combination may be capable of driving cells cycle phases of cholangiocarcinoma cells. Several studies have shown increased susceptibility and also synergistic effects of certain tumor cell stages to various treatments[17-20] and those results seem to be comparable to those in our study. The cell death mechanisms of our study could not be elucidated. However, mechanisms such as increased TNFα receptor I expression leading to programmed cell death activation in association with caspases enzymes activation are possible.

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