Near Eradication of Clinically Relevant Concentrations of Human Tumor Cells by Interferon-Activated Monocytes *In Vitro*

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We have previously reported that low concentrations of interferon (IFN)-activated monocytes exert near-eradicative cytotoxic activity against low concentrations of several human tumor cells *in vitro*. In the present study, we examined 7 human tumor cell lines and 3 diploid lines in the presence or absence of 10 ng/mL IFN α 2a and monocytes. The results confirmed strong cytotoxic activity against 4 of 7 tumor lines but none against 3 diploid lines. To model larger *in vivo* tumors, we increased the target cell concentration and determined the concentration of IFN α 2a and monocytes, required for cell death. We found that increasing the tumor cell concentration from 10- to 100-fold (10^5 cells/well) required an increase in the concentration of IFNs by over 100-fold and monocytes by 10-fold. High concentrations of monocytes could sometimes kill tumor or diploid cells in the absence of IFN. We may conclude that killing of high concentrations of tumor or diploid cells required high concentrations of monocytes that could sometimes kill in the absence of IFN. Thus, high concentrations of tumor cells required high concentrations of IFN and monocytes to cause near eradication of tumor cells. These findings may have clinical implications.

Introduction

INTERFERONS (IFNs) have been shown to exhibit antiviral, antiproliferative, and immunomodulatory effects (Isaacs 1963; Gresser 1982; Bekisz and others 2004). Activated monocytes incompletely kill tumor cells both *in vitro* and *in vivo* (Chen and Koren 1985; Martinet and others 1992; Galligioni and others 1993; Khammari and others 2007). We have previously reported that low concentrations (10^5 per microtiter well) of IFN-activated monocytes exert near-eradicative cytotoxic activity against low concentrations of a number of human tumor cells *in vitro* (10^{3-4} per well) (Baron and others 2007). Diploid cells are resistant in these conditions and may lack monocyte-signaling molecules (Utsugi and others 1991; Galligioni and others 1993; Shi and others 2005; Baron and others 2007). To model the high tumor cell concentrations found *in vivo*, we increased the target cell concentration 100-fold (10^5 cells/well) and determined the concentration of monocytes, with and without IFNs, required for target cell eradication.

Materials and Methods

Tumor cells were cultured in RPMI 1640 containing 10% fetal bovine serum, 1% L-glutamine, and 50 units/mL of

penicillin and streptomycin. Diploid cells were cultured in Eagle's minimum essential media (EMEM) containing 10% fetal bovine serum, 1% L-glutamine, and 50 units/mL of penicillin and streptomycin. Elutriated monocytes were obtained from healthy adult donors by the National Institutes of Health Blood Bank using the Gambro Elutra method (Chen and others 2008). The preparation contained over 90% monocytes, <10% neutrophils, and <5% lymphocytes. As reported earlier, the level of contaminating lymphocytes and neutrophils in the monocyte preparation do not contribute to the antitumor activity observed (Schneider and others 2003; Otten and others 2005; Baron and others 2007). IFN α 2a was obtained from Hoffmann LaRoche (Nutley, NJ). IFN γ was obtained from Intermune Pharmaceutical (Brisbane, CA). A549 lung, human osteosarcoma, LOX melanoma, SNB19 brain tumor, and OVCAR3 ovarian tumor lines were provided by the Developmental Therapeutics Program NCI/NIH and are listed among the 60 human cancer cell lines (NCI 60) maintained by NCI-Frederick. IgrOV1 ovarian tumor line was provided by Dr. Raj Puri of the Center for Biologics Evaluation and Research at the FDA. Diploid lines MRC5, WI38, and FS-4 were purchased from ATCC (Manassas, VA).

We treated low (10^{3-4} per well) in 0.1 mL and high (10^5) concentrations of tumor and diploid cells, with low (10^5 per well) or high (10^6) concentrations of monocytes with and

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without IFN, and then quantified cell death after 3–5 days of incubation by measuring target cell confluence and dye uptake. Specifically, the cells were plated in 96-well plates at varying concentrations and were incubated at 37°C for 4 h to attain adherence. The cells were then treated with varying concentrations of monocytes and IFN. After 3–5 days, the plates were decanted, washed, and stained with crystal violet (2.3% in ethanol) to quantify cell death by dye uptake, colony counts, and/or microscopic percent confluence. Fold inhibition was calculated by comparison with medium control cultures (Baron and others 2007). As we reported previously, in a comparison of colony counts, visual confluence, and crystal violet or [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) vital dye uptake (Baron and others 2007), the live target cells both adhere to the plastic and are stained, whereas monocytes and all dead target cells are washed away during the staining procedure. In the present study, to measure crystal violet dye uptake, 0.1 mL ethanol was added to each well to dissolve the crystal violet. Dye absorbance was measured at 570 nm using a VICTOR³ plate reader (Perkin Elmer, Waltham, MA).

Results

Killing of high concentrations of OVCAR3 tumor cells by monocytes requires higher concentrations of IFNs

Generally, an increased concentration of tumor cells is reported to require stronger antitumor treatments to achieve tumoricidal effects (Khammari and others 2007). To determine whether increased concentrations of OVCAR3 cells would similarly require higher concentrations of IFNs to activate the monocytes' cytotoxic activity, we treated ~10-fold incremental concentrations of OVCAR3 cells with 10-fold increments of IFN α 2a or IFN γ in the presence of 10^5 monocytes/well. After incubation for 4 days, the unattached monocytes were washed away and the adherent tumor cells were stained with crystal violet to determine the 50% cell death endpoint. The findings were confirmed by microscopic determination of confluency and, in some experiments, by MTT assay. IFN- α 2a and γ -activation of monocytes consistently achieved at least 10-fold higher tumor killing than IFN- α 2a or γ alone (data not shown). Further, monocytes alone exhibited only partial (<45%) cell killing for 2×10^3 OV3/0.1 mL in microtiter wells and had no visible effect on 1×10^4 or 1×10^5 OVCAR3 cells/well (data not shown). As shown in Fig. 1, increasing the OVCAR3 tumor cell concentration from 10^{3-4} to 10^5 cells/well strongly increased the concentration of IFNs needed to achieve 50% cell death. The IFN alone control and the monocyte alone control showed no cell inhibition. This finding indicates that to effectively kill higher concentrations of tumor cells, substantially higher concentrations of IFN and/or monocytes are required.

Expanded studies of in vitro modeling of low concentrations of target cells (10^{3-4} per well) and low concentrations of monocytes (10^5 per well)

We confirmed and expanded the previous study by using low concentrations of monocytes and target cells (Baron and others 2007) to include a total of 7 human tumor cell lines and 3 diploid lines in the presence or absence of 10 ng/mL

FIG. 1. Killing of high concentrations of OVCAR3 tumor cells by monocytes requires higher concentrations of IFNs. The interferon alone and the monocyte alone controls showed no cell inhibition. * $P < 0.01$ by Student's t -test comparing 1×10^5 OVCAR-3 concentrations with 2×10^3 and 1×10^4 . IFN, interferon.

IFN α 2a and the stated number of monocytes. In these experiments, the 10 ng/mL IFN α 2a alone demonstrated ~50% killing for the OVCAR3 ovarian tumor cell line and no killing for the other tumor and diploid lines (data not shown). Monocytes alone resulted in 45%–75% killing of 3 of 7 tumor lines, but none of the 3 diploid lines were killed (Table 1). Also, as shown in Table 1, addition of IFN α 2a to the monocytes greatly increased the killing ($P < 0.01$) of the 3 sensitive lines and one insensitive line to near-eradicative levels but did not kill the diploid lines. IFN alone showed no killing. Thus, these expanded studies of killing involving low

TABLE 1. *IN VITRO* MODELING OF LOW CONCENTRATIONS OF TARGET CELLS (10^{3-4} PER WELL) AND LOW CONCENTRATIONS OF MONOCYTES (10^5 PER WELL) ON CELL VIABILITY

Target cells	Monocytes alone treatment		Monocytes + IFN α 2a treatment	
	% Killing	Fold killing	% Killing	Fold killing
OVCAR3 ovarian	75 ^a	4 ^a	99 ^a	100 ^a
Osteosarcoma	50	2	98 ^a	50 ^a
A549 lung	45	2	95 ^a	20 ^a
LOX melanoma	0	0	99 ^a	100 ^a
IgrOV1 ovarian	0	0	0	0
Huh7 liver	0	0	0	0
SNB19	0	0	0	0
WI38 diploid	0	0	0	0
MRC5 diploid	0	0	0	0
FS-4 diploid	0	0	0	0

^a $P < 0.01$ by Student's t -test comparing the variously treated cell lines with their appropriate interferon and monocyte control cultures.

IFN, interferon.

concentrations of monocytes, IFN α 2a, and target cells confirmed and extended earlier studies reporting near eradication of several tumor lines, but not diploid lines.

Effect of high concentrations of target cells (10^5 per well) and high concentrations of monocytes (10^6 per well) on cell killing

High concentrations of monocytes (10^6 per well) without IFN achieved near eradication of all tumor lines tested and overcame resistance to killing by the IgrOV1 ovarian and the SNB19 glioblastoma cell lines and by 1 of 2 diploid lines (Table 2). The addition of IFN α 2a could not enhance the already complete killing by monocytes alone but did enhance killing of 1 diploid cell line. Viabilities of both tumor cells and monocytes were confirmed by colony count and MTT colorimetric cell viability assay, respectively (data not shown).

Discussion

We reported that low concentrations of IFN-activated monocytes (10^5 cells per microtiter well) exerted strong cytotoxic activity against low concentrations (10^{3-4} tumor cells per microtiter well) of some human tumor cells *in vitro*. To model larger *in vivo* tumors, we increased the target cell concentration 100-fold (10^5 tumor cells) and determined the concentration of monocytes, with and without IFN α 2a required for cell death. We found that increasing the tumor cell concentration from 10- to 100-fold increased the required cytotoxic concentration of IFNs by over 100-fold and the required concentration of monocytes by 10-fold. Unexpectedly, killing of tumor cells at higher concentrations of monocytes did not require activation by IFN. Also unexpectedly, at the 10-fold higher concentration of monocytes, the 2 diploid cell lines studied were sensitive to the cytotoxic activity of monocytes, but activation by IFN was needed to kill 1 of the 2 diploid lines. These complex results at the high concentrations of target cells and monocytes suggest that many unidentified variables involving monocytes, IFNs, and tumor cells have complex interactions that need to be identified. Despite these unidentified variables, some biological conclusions can be made, such as, (a) for the first time, activated monocytes achieved near eradication of high concentrations of tumor cells, (b) activation by IFNs strongly

increased monocyte cytotoxic activity under many conditions, and (c) cell death of 2 diploid lines studied occurred only at the highest concentration of monocytes.

Possible mechanisms of cytotoxic activity by monocytes

A number of mechanisms by which monocytes kill tumor cells have been identified. These include cytokines such as tumor necrosis factor, proteases, oxidative compounds such as nitric oxide and peroxides, TRAIL, and thymidines (Drysdale and others 1988; Pak and Fidler 1991; Vanderheyde and others 2004; Tsuno and others 2009). However, the pathways of killing of tumor cells by IFN-activated monocytes have not been determined (Kopper and Lapis 1985). We plan such studies in the future.

Some possible therapeutic approaches using activated monocytes

Our findings show the unanticipated eradication antitumor potency of homologous human monocytes, compared with the previously reported 30%–80% tumor cell inhibition (Manna and Mohanakumar 2002; Vanderheyde and others 2004). An initial consideration is the safety of large numbers of autologous monocytes needed for antitumor effects. The safety of human monocytes from blood banks has been reported in several phase 1 human trials (Faradji and others 1991a, 1991b; Hossne and others 2009). In addition to the safety of monocytes, FDA-approved IFNs are available for clinical studies (Bekisz and others 2010). Thus, studies of clinical antitumor efficacy of IFN-activated monocytes seem feasible and relevant.

As the contact of activated monocytes with tumor cells is important, an initial clinical study of IFN-activated monocytes might be done in patients with tumors localized to the skin and accessible by injection. Subsequent clinical trials of tumors localized to the brain might be considered.

Additional future studies might compare activation by IFNs with other strong activators such as cytosine-phosphate-guanine (CpG) and lipopolysaccharide (LPS) (Manna and Mohanakumar 2002; Sugiyama and others 2005) for antitumor potency. Other studies might evaluate the addition of monocyte-recruiting cytokines such as interleukin-8, interleukin-18, and monocyte chemoattractant protein-1 (MCP-1) (Papadopoulos and others 2008; Ruth and others 2010) to IFN-activated monocytes to determine whether recruiting activated monocytes to tumor sites *in vivo* can provide protection. Importantly, the recruiting cytokines can often also function as activators of monocytes in addition to recruiting (Drysdale and others 1988). Another important study might be to determine whether circulating monocytes, injected intravenously, can migrate to metastatic sites and provide protection. Many of these questions can be addressed in mouse models of human tumors (Kioi and others 2006). Preclinical studies in this mouse model have been started.

Can monocytes provide natural surveillance against tumors?

An important question is whether the strong cytotoxic activity of IFN-activated monocytes reflects the existence of a natural surveillance mechanism against some tumors. Unfortunately, this possibility is not supported by our findings

TABLE 2. *IN VITRO* MODELING OF HIGH CONCENTRATIONS OF TARGET CELLS (10^5 PER WELL) AND HIGH CONCENTRATIONS OF MONOCYTES (10^6 PER WELL) ON CELL VIABILITY

Target cells	Monocytes alone treatment		Monocytes + IFN α 2a treatment	
	% Killing	Fold killing	% Killing	Fold killing
OVCAR3 ovarian	99 ^a	100 ^a	99 ^a	100 ^a
A549 lung	98 ^a	50 ^a	98 ^a	50 ^a
IgrOV1 ovarian	98 ^a	50 ^a	98 ^a	50 ^a
SNB19 glioblastoma	95 ^a	20 ^a	95 ^a	20 ^a
WI38 diploid	98 ^a	50 ^a	98 ^a	50 ^a
MRC5 diploid	0	0	98 ^a	50 ^a

^a $P < 0.01$ by Student's *t*-test comparing the variously treated cell lines with their appropriate control cultures.

and the literature. Against the possibility that monocytes within tumors are activated, there is the finding that most tumors do not produce activators of monocytes (Wojtowicz-Praga 1997; Mocellin and others 2001). Actually, many tumors produce inhibitors of monocytes (Mocellin and others 2001; Dirkx and others 2006). In addition, the concentrations of monocytes and macrophages that naturally infiltrate tumors are generally too low to cause eradication (An and others 1987; Yamashiro and others 1994; Ohta and others 2003; Baron and others 2007). Further, the concentration of tumor-infiltrating macrophages is often correlated not with antitumor protection but rather with angiogenesis and poor prognoses (Dirkx and others 2006; Shieh and others 2009). Thus, available evidence favors the interpretation that monocytes and macrophages do not function as natural surveillance mechanisms against tumors. However, our studies indicate that normal, circulating, nonactivated, autologous monocytes are available for therapeutic activation in clinical trials.

Conclusions

This study is the first report of the conditions for near eradication of a number of human tumors in the presence of human monocytes and IFN. *In vivo* studies in murine models of human tumors have been planned.

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Author Disclosure Statement

No competing financial interests exist.

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