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Murine hypodense eosinophils induce tumour cell apoptosis by a granzyme B-dependent mechanism

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Abstract Purpose: Eosinophils have been shown to potentiate anti-tumour cytotoxicity in both clinical and animal studies. The mechanism by which eosinophils induce tumour cell damage, however, has largely been speculative. The purpose of this study was to identify the mechanisms involved in eosinophil-induced tumour cell cytotoxicity. **Methods:** To investigate eosinophil cytotoxicity, eosinophils were isolated from the peritoneal cavity of *Mesocestoides corti*-infected BALB/c mice, and were separated into normodense (ND) and hypodense (HD) populations using discontinuous Percoll density gradient centrifugation. The tumoricidal activity of ND and HD eosinophils was assessed using the [^{51}Cr]-release cytotoxicity assay (a measure of cytolytic activity) and the JAM assay (a measure of apoptotic activity). Investigation of apoptosis-inducing molecules in HD eosinophils was undertaken by RT-PCR. The calcium chelator EGTA, serine protease inhibitor aprotinin and a competitive substrate for granzyme B were used to assess the role of perforin and granzyme B in HD eosinophil killing. **Results:** Cytotoxic activity induced by HD eosinophils was significantly greater than that of ND eosinophils, and apoptosis was the principal killing mechanism. RT-PCR analysis revealed that HD eosinophils express mRNA for perforin, granzyme B and Fas ligand. Furthermore, HD eosinophil killing was markedly inhibited by EGTA, intracellular aprotinin and the granzyme B competitive substrate. **Conclusions:** These data are consistent with a hypothesis that murine HD eosinophils elicit tumoricidal activity via a granzyme B-dependent mechanism.

Key words Hypodense · Eosinophil · Apoptosis · Granzyme B · Perforin

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Introduction

The local presence of eosinophils has long been noted in association with neoplastic conditions [1], and several clinical reports have suggested a correlation between tumour-associated eosinophilia and increased survival time [2–5]. It has recently been reported that IL-2 immunotherapy for neoplastic disease induces peripheral eosinophilia [6–8]. Indeed, circulating eosinophils isolated following IL-2 therapy express the hypodense (HD) phenotype [6–8]. HD eosinophils exhibit enhanced biological activity, including augmented cytotoxicity towards parasites, increased FcεR expression, and upregulated release of superoxide, LTC_4, PAF and EPO [reviewed in 9]. Moreover, eosinophils isolated from IL-2-treated cancer patients demonstrate enhanced in vitro cytolytic activity against tumour targets both in the presence and absence of tumour-specific antibody [6]. Taken together, these data indicate that eosinophils may contribute to current and future cancer immunotherapeutic regimens. The data also suggest that a normodense (ND) to HD transition may lead to the acquisition of eosinophil tumoricidal activity. To date, however, few studies have directly addressed whether, or how, eosinophils limit tumour growth.

Although the killing mechanism of eosinophils towards tumour cells remains undetermined, the four main eosinophil granular proteins, eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), major basic protein (MBP), and eosinophil-derived neurotoxin (EDN), have all been shown to induce tumour cell cytolysis in vitro [10–12]. EPO deposition has also been described

in vivo in association with certain lymphoid malignancies [13], and correlations have been noted between MBP deposition and tissue damage in lymph nodes of Hodgkin's disease patients [14]. A more recent study revealed a significant increase in serum levels of ECP, EPO and EDN in cancer patients as compared to healthy volunteers [15]. Although these studies indirectly suggest the involvement of these toxic molecules in an eosinophil-mediated anti-tumour response, no study to date has directly examined their role in tumour cytotoxicity induced by intact eosinophils. Furthermore, it has recently been shown that supernatants obtained from circulating eosinophils isolated from patients undergoing IL-2 cancer immunotherapy failed to kill allogeneic renal carcinoma or melanoma cells in vitro, despite the presence of EDN and ECP [16]. This suggests that alternative mechanisms may be operative in the eosinophil tumoricidal response.

In this study, we address the mechanism(s) by which mouse eosinophils kill syngeneic tumour cells in vitro, and demonstrate that HD eosinophils isolated from *Mesocestoides corti*-infected mice kill syngeneic A20 B cell lymphoma cells by apoptosis, but not by cytolysis. RT-PCR analysis for well characterized apoptosis-inducing molecules revealed that parasite-elicited HD eosinophils express perforin, granzyme B and FasL. Calcium chelation, serine protease inhibition and competitive inhibition of granzyme B, but not blocking of TNF-α activity, significantly inhibited HD eosinophil tumoricidal activity. These data implicate perforin and granzyme B in the cytotoxic activity of HD eosinophils, a novel finding which elucidates, at least in part, the mechanisms by which eosinophils could be involved in anti-tumour immunity.

Materials and methods

Animals

The BALB/c mice utilized for this study were purchased from Charles River Laboratories (Montreal, Que.) at 6–8 weeks of age. Animals were housed in pathogen-free conditions under CCAC guidelines, and allowed food and water ad libitum.

Parasite infection

Tetrathyridia of the tapeworm *Mesocestoides corti* were kindly provided by Dr. M. Novac (University of Manitoba, Winnipeg, Man.). Parasites were maintained by IP passage in CD1 mice. For parasite-induced eosinophilia, female BALB/c mice, 7–10 weeks old, received 100 µl packed tetrathyridia in 300 µl sterile phosphate-buffered saline containing 100 U/ml penicillin G and 100 µg/ml streptomycin (Gibco, Burlington, Ont.) (PBS-PenStrep) intraperitoneally (i.p.) through an 18-G needle.

Eosinophil purification

Peritoneal lavage was performed 24 days following parasite infection, using four separate washings with a total of 15 ml chilled (4 °C) sterile PBS-PenStrep supplemented with 5% fetal bovine serum (v/v) and 10 U/ml heparin (Organon Teknika, Scarborough, Ont.). The lavage was left for approximately 5 min, to allow the parasites to settle out, and the cells were removed. The sedimented material was resuspended in PBS-PenStrep and again allowed to settle for 5 min to allow any co-sedimenting cells to be removed from the parasites. This was repeated three more times, and cells obtained from all these suspensions were pooled. Cells were pelleted by centrifugation at $200 \times g$ for 10 min at room temperature, and were washed once in RPMI-1640 supplemented with 5% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µM 2-mercaptoethanol, 20 mM HEPES and 2 mM L-glutamine (Gibco, Burlington, Ont.). Cells were suspended in 25 ml PBS-PenStrep with 4 mM EDTA and 0.1% (w/v) bovine serum albumin (BSA; Sigma, Mississauga, Ont.).

100% Percoll stock was prepared by adjusting Percoll (Pharmacia Biotech, Baie D'Urfe, Que.) to isotonicity with $10\times Ca^{2+}Mg^{2+}$-free HBSS and adding HEPES to a final concentration of 10 mM. The pH was adjusted to approximately 7.4 with 1 M HCl. Three densities of Percoll were made: 1.0642 g/ml (50%), 1.0749 g/ml (59%), and 1.0833 g/ml (66%), by diluting Percoll stock with 0.15 M sterile NaCl. Discontinuous Percoll gradients were prepared by careful pipetting of 3 ml each dilution into sterile 15 ml conical centrifuge tubes. A maximum of 3.0×10^7 cells were layered on top of the Percoll gradients, and the gradients were spun at $400 \times g$ for 30 min at room temperature. Cells from the 50–59% and 59–66% interface were removed. This entire procedure was performed under sterile conditions.

Following three washes with RPMI-1640, cells were placed in tissue culture flasks with RPMI-1640 supplemented with 10% FBS for 1 h at 37 °C to remove adherent cells. This process was repeated once with a fresh flask. Non-adherent cells from the 50–59% and the 59–66% Percoll interfaces were designated as HD eosinophils and ND eosinophils, respectively. All cells were subsequently washed twice with RPMI-1640, and cell viability was assessed by trypan blue exclusion (> 95% viability in all experiments). Cytospin (Shandon Instruments) preparations were stained with May-Grünwald and Giemsa stains (BDH) for cell differentials, and all eosinophil populations were determined to be > 95% eosinophils (< 1% lymphocytes) by counting > 200 cells in random fields under oil immersion.

Cytotoxicity assays

^{51}Cr-release and JAM assays were performed as described elsewhere [17, 18] with slight modifications. Briefly, for ^{51}Cr-release assays, A20 B cell lymphoma cells (BALB/c origin, ATCC) were labelled with 200–400 µCi [^{51}Cr]-Na_2CrO_4 in PBS-PenStrep for 1 h at 37 °C with frequent agitation. Cells were washed twice in RPMI-1640 supplemented with 5% FBS by centrifugation at $200 \times g$ for 7 min, and were incubated in RPMI-1640 supplemented with 10% FBS for an additional 1 h at 37 °C. After a separate washing to remove free ^{51}Cr, tumour cells were then plated in triplicate in RPMI (supplemented with 10% FBS) at a density of 1×10^4 cells/well with effector cells. Following 18 h incubation (37 °C), cells were pelleted by centrifugation at $200 \times g$ for 3 min, and 100 µl supernatant was removed for reading in a Wallac 1480 γ-counter. The cytolytic activity of effector cells was determined by the following calculation:

$$\%\ \text{lysis} = \left(\frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}}\right) \times 100 \quad \text{(a)}$$

For the JAM assay, A20 tumour cells were labelled with 5–10 µCi/ml [^{3}H]-thymidine for 6 h. The cells were washed three times as above, and plated (in quadruplicate) with effector cells identical to above. Four aliquots were harvested immediately (Skatron Instruments) onto glass filtermats, to represent total incorporated label in intact DNA at time zero. Eighteen hours later, cells were harvested and assessed for remaining intact DNA using an automatic Wallac 1410 scintillation counter. Apoptotic activity was calculated using the following equation:

$$\%\ \text{DNA damage} = \left(\frac{T_{18} - \text{experimental}}{T_{18}}\right) \times 100 \qquad \text{(b)}$$

where T_{18} refers to counts per minute (cpm) of labelled control cells at 18 h and *experimental* refers to cpm from labelled cells incubated with effector cells at 18 h.

For inhibition of killing assays, the granzyme B competitive substrate (GBCS, benzyloxycarbonyl-Ala-Ala-Asp-CH_2Cl) was purchased from Cedarlane Laboratories, the polyclonal rabbit anti-mouse TNF-α antiserum (Genzyme) was generously provided by Dr. J. Marshall (Dalhousie University, Halifax, N.S.), and normal rabbit serum was provided by Dr. R.I. Carr (Dalhousie University, Halifax, N.S.).

Reverse-transcriptase polymerase chain reaction

RNA was obtained by re-suspending up to 1×10^7 cells in 1 ml TRIZOL reagent (Gibco/BRL), and was extracted essentially as described in the manufacturer's recommendations. RNA (1 μg) was transcribed to cDNA using M-MLV RT enzyme, as described in the manufacturer's instructions. Amplification was carried out using an automated thermocycler (MJ Research). Intron-spanning primers for FasL, perforin and granzyme B (Gibco/BRL) were generously provided by Dr. D.W. Hoskin (Dalhousie University, Halifax, N.S.), and β-actin primers were supplied by Dr. A. Stadnyk (Dalhousie University). Primer sequences were as follows:

granzyme B: 5′ = GCCCACAACATCAAAGAACAG,
3′ = AACCAGCCACATAGCACACAT
FasL: 5′ = ATGGTTCTGGTGGCTCTGGT,
3′ = GTTTAGGGGCTGGTTGTTGC
perforin: 5′ = TCAATAACGACTGGCGTGTGG,
3′ = GTGGAGCTGTTAAAGTTGCGG;
β-actin: 5′ = CTGGAGAAGAGCTATGAGC,
3′ = TTCTGCATCCTGTCAGCAATG

Products were amplified using 35 cycles with the following conditions: 94 °C (30 s), 61 °C (30 s) and 72 °C (30 s) for β-actin, perforin, and granzyme B.

TNF-α mRNA was measured using a CytoXpress detection kit (Biosource International, Montreal, Que.). Products were separated on 1.5% agarose containing 750 ng/ml ethidium bromide, and visualized by exposure to UV light on a transilluminator.

T cell isolation and anti-CD3 activation

T cell preparations were obtained from the spleens of C57BL/6 mice sensitized 7 days earlier with an i.p. injection of 5×10^7 mitomycin C-treated BALB/c spleen cells. T cells were enriched, as we have described previously [19], by passage through nylon wool and complement depletion of Mac-1^+ and $B220^+$ cells, and were routinely ≥ 90% T cells (by flow cytometry using anti-Thy 1.2, Cedarlane Laboratories, Hornby, Ont.). Cells were activated for 48 h with hamster anti-mouse CD3ε (clone 145–2C11, ATCC) prior to use in cytotoxicity assays.

Protein-loading of target cells

Target cells were loaded with aprotinin (Sigma, Mississauga, Ont.) or BSA using the osmotic lysis of pinosomes technique, modified from Nakajima and Henkart [20]. Briefly, [^{3}H]-thymidine-labelled tumour cells were pelleted and suspended in 500 μl serum-free RPMI-1640 containing 0.5 M sucrose, 10% (w/v) polyethylene glycol 1000 (Sigma) and 10 mg/ml aprotinin (or BSA) for 10 min at 37 °C. Pre-warmed hypotonic RPMI-1640 (60%) was added, cells were incubated for 2 min at room temperature, and were washed twice with RPMI-1640 supplemented with 5% FBS. Loaded cells were subsequently used in cytotoxicity assays as described above.

Results

Eosinophil tumoricidal activity

Cytotoxic activity of eosinophils against the syngeneic A20 B cell lymphoma cell line was assessed using the 18 h ^{51}Cr-release cytotoxicity assay (cytolysis) or the JAM assay (apoptosis). Very little to no cytolytic activity was detected by either ND or HD eosinophils (Fig. 1A) using the ^{51}Cr-release cytolytic assay, although HD eosinophils were marginally more efficient. Conversely, a substantial amount of apoptotic killing was detected in tumour targets incubated with HD eosinophils (Fig. 1B), while ND eosinophils were much less efficient apoptotic killers (23.2 ± 3.7% HD vs. 9.5 ± 2.4% ND at 100:1 E:T, $P < 0.001$). In addition to revealing that HD eosinophils are more effective tumour killers than ND eosinophils, these data suggested that the primary killing mechanism by eosinophils involves an apoptotic pathway rather than cytolytic mechanisms.

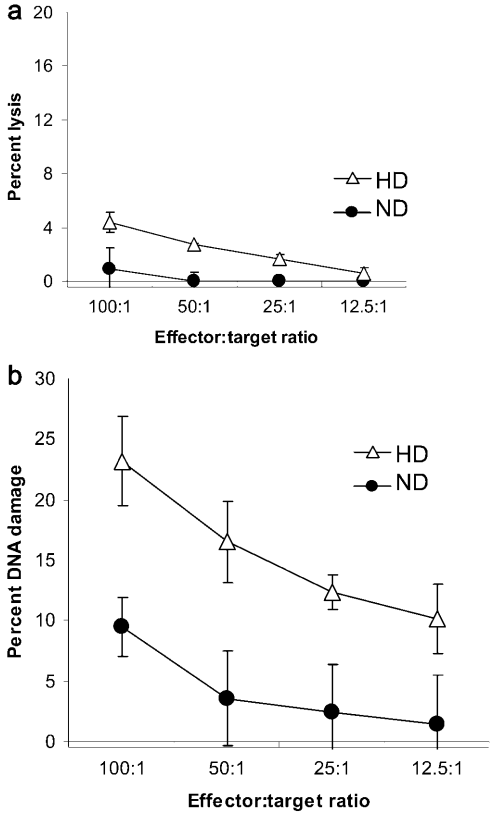

Fig. 1A–B Cytotoxicity of ND (●) and HD (△) eosinophils towards A20 tumour targets. **A** ^{51}Cr-release was used as a measure of eosinophil cytolytic activity in an 18 h assay. Little cytolysis was detected by either eosinophil population. Results are representative of nine separate experiments. **B** DNA damage was assessed using the JAM technique as a measure of eosinophil-induced apoptosis, also in an 18 h assay. Although only little DNA damage was detected in ND eosinophils even at the highest E:T ratio, substantial target cell apoptosis was induced by HD eosinophils. Results are representative of 12 separate experiments

Expression of apoptosis-inducing molecules in HD eosinophils

To examine the expression of apoptosis-inducing cytotoxic mediators in HD eosinophils, RT-PCR was performed using intron-spanning primers for perforin, granzyme B, and FasL. We consistently ($n = 6$) found that HD eosinophils express significant levels of mRNA for perforin, FasL and granzyme B (Fig. 2). No TNF-α mRNA was expressed by HD eosinophils, as demonstrated in two separate experiments using a quantitative TNF-α RT-PCR kit. These studies revealed fewer than 2000 copies of TNF-α mRNA isolated from 3×10^6 HD eosinophils (data not shown), suggesting that TNF-α was unlikely to be involved in the cytotoxic response. Since only HD eosinophils consistently demonstrated the ability to induce apoptosis in tumour cells, we compared levels of granzyme B, perforin and FasL in HD and ND eosinophils. In several experiments using semi-quantitative RT-PCR and densitometric analysis, we were unable to demonstrate a significant difference between the two eosinophil subsets (data not shown), indicating that the regulation of activity, rather than mRNA expression, of these mediators is what differentiates the two populations with respect to tumour killing.

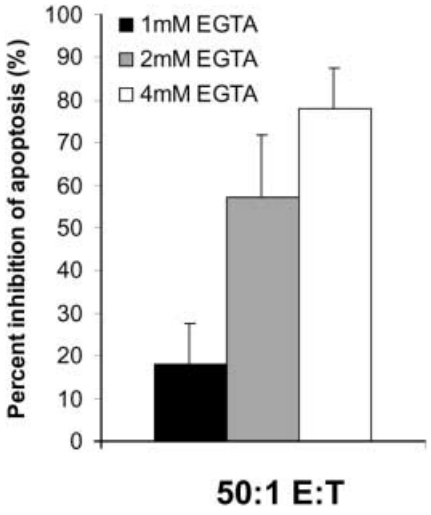

Fig. 3 Effect of calcium chelation on HD eosinophil-induced tumour cell apoptosis. 18 h JAM assay was performed in the presence of 1–4 mM EGTA + 0.75–3 mM $MgCl_2$ to assess the role of perforin in HD eosinophil cytotoxicity. This study demonstrates that EGTA inhibits HD tumoricidal activity in a dose-dependent manner. Results are presented as mean inhibition (%) of killing ± SEM of three separate experiments with 1 mM EGTA (+ 0.75 mM $MgCl_2$, *closed bar*), four experiments with 2 mM EGTA (+ 1.5 mM $MgCl_2$, *speckled bar*), and five experiments with 4 mM EGTA (+ 3 mM $MgCl_2$, *open bar*)

Mechanism of HD eosinophil killing

Given the finding that HD eosinophils both express perforin and granzyme B, and also appear to mediate tumour cell damage by an apoptotic pathway, we next assessed the role of these molecules in tumour killing by HD eosinophils. To examine the role of granzyme B in tumour killing, we inhibited perforin polymerization with EGTA, as perforin polymerization is a requisite first step in granzyme B apoptosis. The results (Fig. 3) demonstrate that in the presence of 1–4 mM EGTA, HD eosinophil-induced tumour cell apoptosis is significantly inhibited in a dose-dependent manner. Indeed, inhibition reached nearly 80% at the highest concentration of EGTA used ($P < 0.01$).

Inhibition of perforin polymerization is an indirect method of investigating granzyme B-mediated killing. To more directly assess the involvement of granzyme B, aprotinin was used to inhibit serine protease activity. Inhibition of granzyme B activity by intracellular loading of target cells with aprotinin has been shown by others to block apoptosis mediated by CTL and CTL granzymes [20]. We first confirmed that aprotinin could inhibit T cell cytotoxicity (an indirect assay of granzyme B inhibition) using anti-CD3ε-stimulated T cells obtained from C57BL/6 spleens following sensitization to BALB/c spleen cells. At both 4 h and 18 h, intracellular aprotinin significantly inhibited apoptosis induction by CTL as compared to the BSA control ($P < 0.01$ at E:T of 12:1 and 6:1, data not shown). We then examined the effect of loading tumour targets with aprotinin (Ap) on HD eosinophil mediated killing (Fig. 4), and found that Ap-loaded tumour targets were completely insensitive to HD eosinophil-mediated killing, as compared to BSA loaded intracellularly ($P < 0.0001$).

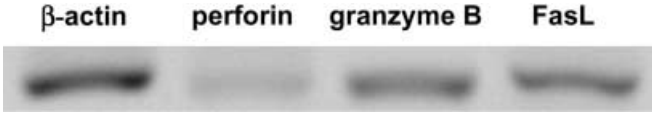

Fig. 2 Expression of cytotoxic molecules by eosinophils. Semi-quantitative RT-PCR was performed on HD eosinophils. Results show that HD eosinophils transcribe message for perforin, FasL and granzyme B. Results are representative of four separate experiments

This experimentation demonstrated that serine protease activity is required within the target cell cytoplasm for HD eosinophil-induced apoptosis, which is consistent with a role for granzyme B. However, serine protease inhibitors have been shown elsewhere to also inhibit TNF-α-induced apoptosis [21]. Because human eosinophils have been shown to produce TNF-α [22], it was important to confirm that TNF-α, despite our inability to detect its message by quantitative RT-PCR, did not contribute to HD eosinophil-induced apoptosis. In three separate experiments, rabbit anti-mouse TNF-α antiserum used at a concentration previously shown to completely inhibit rat TNF-α-mediated apoptosis in the TNF-α sensitive cell line L929 (Jean Marshall, personal communication) failed to have a significant effect on HD-induced cytotoxicity (data not shown), confirming that TNF-α was not involved in HD eosinophil-induced killing in our model.

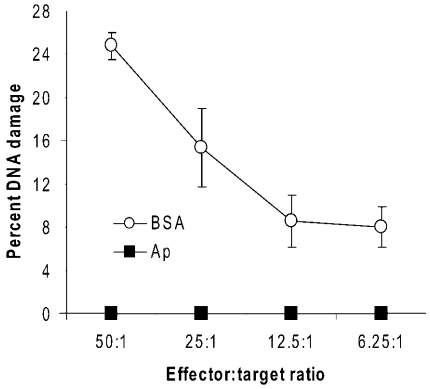

Fig. 4 Effect of serine protease inhibition on HD eosinophil tumoricidal activity. Following confirmation that aprotinin inhibits T cell cytotoxicity in both 4 h and 18 h assays (data not shown), tumour cells were loaded intracellularly with aprotinin (■) or BSA (○) as a control, and assessed for DNA damage by JAM assay. Aprotinin-loaded targets were completely insensitive to HD eosinophil-induced DNA damage at 18 h

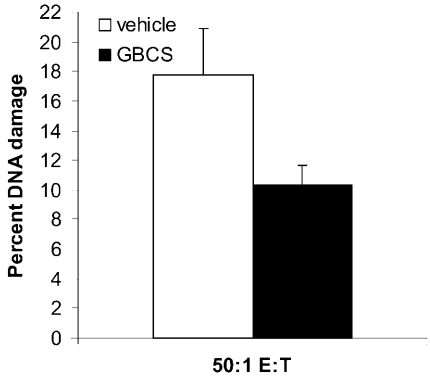

Fig. 5 The role of granzyme B in HD eosinophil-induced tumour apoptosis. 18 h JAM assay of HD eosinophil killing was performed with vehicle alone (*open bars*), or granzyme B competitive substrate (GBCS, 45 μM; *closed bars*). Representative results of three experiments demonstrate a significant ($P < 0.001$) reduction in killing with GBCS compared to vehicle alone

In a final experiment, designed to confirm the role of granzyme B in eosinophil killing of tumour targets, a granzyme B competitive substrate (GBCS, benzyloxycarbonyl-Ala-Ala-Asp-CH_2Cl) was used in cytotoxicity assays. The granzyme inhibitor was used rather than granzyme KO mice for two reasons. First, granzyme KO mice on the BALB/c background are not currently available and second, the use of exogenous inhibitors controls for the potential amplification of redundant pathways often seen in killing studies using KO mice. Cells were incubated with various concentrations of GBCS or vehicle alone (to a final concentration of 0.0002% DMSO in RPMI-1640), and assessed for apoptosis. In preliminary experiments, 45 μM GBCS was determined to be the optimal dose for apoptosis inhibition (data not shown). Figure 5 shows the reduction in DNA damage in one representative experiment of three separate experiments using GBCS. This experiment confirms that aspase activity is involved in the induction of apoptosis in A20 targets by HD eosinophils.

Discussion

There are numerous reports suggesting that eosinophils potentiate tumoricidal activity both in humans [6, 8, 15] and in mouse models [23, 24] of cytokine immunotherapy. Our interest in eosinophil tumoricidal activity stems primarily from reports by Leder's group [23, 24], in which tumour cells engineered to secrete IL-4 were rapidly infiltrated by eosinophils, leading to elimination of the tumours. They further revealed that T cells, B cells, mast cells and NK cells were not required for the anti-tumour response, while granulocyte-eliminating antibody and neutralizing antibody to IL-5 inhibited the response [24]. Interestingly, however, IL-5 on its own is not sufficient to induce anti-tumour immunity by eosinophils when produced locally by tumour cells [25], demonstrating that a factor other than IL-5 is induced by IL-4 to elicit anti-tumour activity by eosinophils. While these studies support reports suggesting a role for eosinophils in anti-tumour immunity in humans, they were limited in that eosinophils could not easily be isolated from the subcutaneous tumour lesion for in vitro confirmation of their tumoricidal properties.

Indeed, there has been little experimentation examining eosinophil cytotoxicity towards tumours. While earlier reports have demonstrated that enriched eosinophil granules potentiate tumour cell cytotoxicity in vitro [11, 12], there is recent evidence that suggests that factors other than the classical eosinophil granules mediate tumour damage in vivo. Eosinophil supernatants from cancer patients undergoing IL-2 therapy, for example, are not capable of inducing tumour cell cytolysis [16], despite the abundance of both ECP and EDN. Consistent with this finding, we were unable to demonstrate cytolytic activity induced by intact eosinophils in standard ^{51}Cr release assays, suggesting that large-scale eosinophil degranulation did not occur. Our findings are in contrast to other reports [6, 12], in which human and guinea pig eosinophils elicit tumour cell cytolysis by the same assay. Although this discrepancy may be due to species differences, it more probably relates to the purity of the eosinophil populations used, as our eosinophil purity was in excess of 95% in all studies, whereas others [6] used eosinophils with a purity threshold of only 75%, thus making attribution of cytolytic activity to a particular cell type difficult. It is also possible that the use of a cestode infection to attract eosinophils into the peritoneal cavity influences the cytotoxic phenotype of eosinophils, although it is difficult to envision how these activated HD eosinophils could be influenced to downregulate, rather than upregulate, cytolytic activity.

In contrast to our finding with cytolytic assays, apoptotic assays demonstrated tumour killing activity by HD, but not ND eosinophils, suggesting that apoptosis is the primary effector mechanism for eosinophil cytotoxicity, and that a ND to HD transition may be

required for this activity. This presents a new paradigm for eosinophil cytotoxicity, as the prevailing theory to date has been that eosinophil-derived toxic granules induce cytolysis, not apoptosis, of tumour targets [13, 26, 27].

Various molecules have been associated with induction of apoptosis in tumour cells, including perforin, granzyme B, Fas L and TNF-α. In this study, we confirmed that perforin, granzyme B and Fas L were all expressed by HD eosinophils, while TNF-α was undetectable. Although Fas L has been demonstrated in human eosinophils [28, 29], perforin and granzyme B expression is widely regarded to be restricted to CTL and NK cells. Despite the fact that TNF-α expression has previously been described in human eosinophils [22], our inability to demonstrate its expression in mouse HD eosinophils is consistent with the failure of neutralizing anti-TNF-α mAb to reduce apoptosis induction (data not shown), confirming that TNF-α is not involved in HD eosinophil tumoricidal activity.

The requirement of these molecules for eosinophil cytotoxicity was initially investigated using the calcium chelator EGTA, as calcium is required to prevent perforin polymerization in target cell membrane [30], and granzyme B delivery depends on perforin polymerization [31]. The finding that EGTA almost completely ablated eosinophil cytotoxic activity (77.7 ± 9.6% inhibition of apoptosis at 4 mM) can be interpreted in different ways, since EGTA also downregulates cellular exocytosis [32] and could thereby deleteriously affect eosinophil mediator release in addition to blocking perforin polymerization. Furthermore, long-term chelation of extracellular calcium could have deleterious effects on eosinophil function by depleting intracellular calcium stores, as has been demonstrated with CTL [33]. Eosinophils, for example, require intracellular calcium mobilization for EPO exocytosis in response to eosinophil agonists [34]. Because of these competing possibilities, the EGTA data fail to conclusively demonstrate a role for perforin in HD eosinophil cytotoxicity, although they suggest that a degranulation pathway is involved.

The association between granzyme B expression and HD eosinophil cytotoxicity was strengthened by the demonstration that both aprotinin and a competitive substrate for granzyme B significantly inhibited tumour cell apoptosis. Intracellular aprotinin inhibits cytotoxicity by CTL, perforin and granzyme A-transfected mast cells [20], and antibody-activated T cells [35], and inhibits enzymatic activity of purified granzyme A and B in vitro [36]. Although aprotinin also inhibits other serine proteases [21], the reduction in cytotoxicity with a competitive substrate for granzyme B corroborates a role for granzyme B in the killing mechanism.

Taken together, the data from the EGTA experiments, the Ap experiments, and the granzyme B competitive substrate experiments, are supportive of a hypothesis that places granzyme B as a central mediator of HD eosinophil-induced tumour cell apoptosis. This is, to our knowledge, the first report of these molecules being involved in murine eosinophil cytotoxicity. Our finding that ND eosinophils also possess mRNA for these cytotoxic mediators, but do not kill, indicates a higher level of control for eosinophil cytotoxicity than was expected.

Substantial circumstantial evidence suggests a role for eosinophils in mediating tumour cell damage in both mice and humans. The work described in this report suggests that mouse eosinophils of the hypodense phenotype kill tumour cells in vitro using an apoptotic pathway dependent on granzyme B. Further, only some targets tested demonstrated sensitivity to eosinophil tumoricidal activity (unpublished observations), suggesting that targeted recognition, rather than non-specific degranulation, is involved in destruction of tumours. These data provide a mechanism by which activated, extravasated, hypodense eosinophils may exhibit anti-tumour activity in cancer, which may assist in the development of novel therapies to better treat human malignancies.

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