Human polymorphonuclear neutrophils specifically recognize and kill cancerous cells

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Abbreviations: BEN, benign ethnic neutropenia; DBL, proto-oncogene DBL; DPI, diphenyleneiodonium; E:T, effector:target; G-CSF, granulocyte-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; GVHD, graft-versus-host disease; H-Ras, Harvey rat sarcoma viral oncogene homolog; mAb, monoclonal antibody; MEK, mitogen-activated protein kinase kinase; mTOR, mammalian target of rapamycin; NADPH, nicotinamide adenine dinucleotide phosphate; NBT, nitroblue tetrazolium; NSCLC, non-small cell lung carcinoma; PI3 kinase, phosphoinositide 3-kinase; PMN, polymorphonuclear neutrophils; Rac1, Ras-related C3 botulinum toxin substrate 1; RhoA, Ras homolog family member A; ROS, reactive oxygen species; TC-21, teratocarcinoma oncogene TC21; TGFβ, transforming growth factor

Introduction

Polymorphonuclear neutrophils (PMNs), the main effectors of the innate immune system, have rarely been considered as an anticancer therapeutic tool. However, recent investigations using animal models and preliminary clinical studies have highlighted the potential antitumor efficacy of PMNs. In the current study, we find that PMNs from some healthy donors naturally have potent cancer-killing activity against 4 different human cancer cell lines. The killing activity appears to be cancer cell-specific since PMNs did not kill primary normal epithelial cells or an immortalized breast epithelial cell line. Transfecting the immortalized mammary cells with plasmids expressing activated forms of the rat sarcoma viral oncogene homolog (Ras) and teratocarcinoma oncogene 21 (TC21) oncogenes was sufficient to provoke aggressive attack by PMNs. However, transfection with activated Ras-related C3 botulinum toxin substrate (Rac1) was ineffective, suggesting specificity in PMN-targeting of neoplastic cells. Furthermore, PMNs from lung cancer patients were also found to exhibit relatively poor cancer-killing activity compared to the cytolytic activity of the average healthy donor. Taken together, our results suggest that PMN-based treatment regimens may represent a paradigm shift in cancer immunotherapy that may be easily introduced into the clinic to benefit a subset of patients with PMN-vulnerable tumors.

Prior investigations have demonstrated that PMNs play a critical role in tumor destruction in murine cancer models.5,6 An elegant study using a strain of naturally cancer-resistant mice demonstrated that PMNs, macrophages, or natural killer cells (but not T cells) from these animals caused regression of established tumors.7 Furthermore, this immunity was transmissible as tumor-susceptible naive mice adoptively transferred with these innate immune cells, including PMNs, became cancer-resistant.8 Our own published studies have also demonstrated that PMNs are among principal effectors in a combined yeast-derived β-glucan and antitumor monoclonal antibody (mAb) cancer immunotherapy.9,10 Finally, in a pilot clinical trial a complement-fixing antibody capable of recruiting PMNs to the malignant lesion was demonstrated to elicit tumoricidal activity via activation of intratumoral PMNs.11 Therefore, these animal and
human studies provide solid experimental evidence to support the premise that PMNs can be cancer-killing effector cells and may potentially be sufficient to destroy tumors.

In the current study, we investigate human neutrophil cancer killing activity and report that neutrophils from some healthy donors have potent, and highly specific, cancer cell killing activity. However, neutrophils from other healthy individuals exhibit a very weak cancer cell cytolysis. We further demonstrate that single, specific oncogenic events (e.g., activated Ras) are sufficient to convert a neutrophil insensitive cell to a neoplastic cell that is vigorously attacked by neutrophils. These findings suggest that naturally occurring defects in neutrophil anticancer activity may facilitate the development of malignancies. Thus, we examined neutrophils from human lung cancer patients and found that their neutrophils had an overall lower tumor cell killing activity relative to that of the average healthy donor. Irradiation of PMNs did not significantly alter their tumoricidal activity. Thus, adoptive transfusion of potent antitumor PMNs from prescreened healthy donors into cancer patients may be an effective approach to transfer immunity against tumors. Alternative strategies may include the reactivation of neutrophils in cancer patients among the sub-population that retains activation-competent PMNs, as well as a tumor whose pattern of molecular transformation confers vulnerability to PMN attack, such as oncogenic Ras.

Results

Neutrophils from healthy donors exhibit potent and specific cancer cell cytotoxicity

We first set out to determine whether neutrophils from healthy donors are capable of killing human cancer cells. To this end, neutrophils from healthy donors were co-cultured with the human cervical cancer cell line Hela, the ovarian cancer cell line SKOV-3, the pancreatic adenocarcinoma cell line Capan-1 and non-small cell lung carcinoma cell line (NSCLC) A549 at different effector to target (E:T) cell ratios. Strikingly, PMNs from 2 independent healthy donors exhibited broad variability in the levels of cytolysis against the 4 cancer cell lines (Fig. 1A). To examine whether neutrophil-mediated killing is specific to cancerous cells, both tumor cells and either normal epithelial cells, or non-transformed, immortalized breast epithelial cells, were used as target cells for PMNs derived from a high activity donor. As shown in Figure 1B, neutrophils from a high activity donor demonstrated potent killing activity against tumor cells with minimal cytotoxicity against normal cells, suggesting that innate neutrophils from a subset of healthy donors have potent and specific cancer cell killing capabilities.

Neutrophils specifically recognize and target certain oncogenically transformed cells

Neutrophils from healthy donors distinguish between transformed cells and normal cells for cancer cell-specific cytotoxicity. To further define the molecular basis of this specificity, we transfected the immortalized but non-transformed human breast epithelial cell line MCF-10A with a panel of different oncogenes and assayed the resultant cell lines for sensitivity to neutrophil-mediated killing. Transfection with plasmids encoding the teratocarcinoma oncogene TC21 and Ras homolog family member A (RhoA) rendered MCF-10A cells vulnerable to PMN-mediated killing (Fig. 2A), as did the Rho exchange factor DBL and the activated form of oncogenic H-Ras (Fig. 2B). However, the presence of activated Ras-related C3 botulinum toxin substrate 1 (Rac1) did not sensitize the cells to PMN-mediated cytotoxicity, seeming in contrast to be somewhat protective (Fig. 2A-B). Under these conditions, PMNs did not kill either empty vector-transfected cells or normal breast epithelial cells.

These results suggested that some oncogenic signaling pathways, but not others, confer PMN vulnerability to cancer cells. To define the nature of these signaling pathways, we treated MCF-10A cells transfected with activated TC21 with a series of specific signaling pathway inhibitors prior to assaying for neutrophil killing. The phosphatidylinositol 3’-kinase (PI3K) inhibitor LY294002 and the p38 kinase inhibitor SB203580 inhibited the action of the neutrophils whereas the MEK inhibitor PD98059 and the mTOR inhibitor rapamycin did not (Fig. 2C). In fact, rapamycin appeared to enhance the cytolytic effect, particularly at high neutrophil doses.

H$_2$O$_2$ catalase from neutrophils plays a critical role in neutrophil-mediated cancer cell killing

PMNs are short-lived leukocytes that are quick to respond and provide the first line of host defense against pathogens through phagocytosis, extracellular degranulation, and spreading of extracellular traps. Neutrophils can release reactive oxygen species (ROS) and produce hydrogen peroxide (H$_2$O$_2$), cytotoxic factors crucial to eradicate bacterial infections. To prospectively examine the machinery by which neutrophils kill malignant cells, the levels of ROS and H$_2$O$_2$ were modified using either catalase, the enzyme catalyzing the decomposition of hydrogen peroxide into water and oxygen, or alternatively, inhibitors of the ROS catalytic enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase were applied to the in the cytotoxicity assay. As shown in Figure 3, addition of catalase significantly decreased PMN-induced A549 lung cancer cell killing, suggesting that H$_2$O$_2$ produced by neutrophils is essential to this immune response. Surprisingly, NADPH oxidase inhibitors, including AG490, apocyanin and diphenyleneiodonium (DPI) did not significantly alter PMN-mediated A549 lung cancer cell cytolysis. In fact, inhibition of NADPH oxidase somewhat increased neutrophil cancer cell-killing activity, implying that neutrophil respiratory burst or super oxide production is not required for the anticancer effect observed.

PMN from human NSCLC patients have significantly decreased superoxide anion production and malignant cell killing potential

In light of our observation that neutrophils can specifically target and kill transformed cells, we reasoned that cancer patients...
may have decreased neutrophil antitumor cytoxic properties, thus leading to disease progression. To address this possibility, healthy donors and human NSCLC patients were recruited to assess their neutrophil killing potential. The nitroblue tetrazolium (NBT) assay has been widely used to measure the production of superoxide anions in PMNs. We found that the basal level of superoxide anion production in PMNs from cancer patients was significantly lower than that of PMNs from healthy donors (Fig. 4A). PMA stimulation can stimulate neutrophils to produce superoxide anions. The superoxide anion production level in healthy donors was also higher than that in cancer patients after both were stimulated. Next, we sought to directly examine PMN-mediated cancer cell killing activity against 3 tumor cell lines. Neutrophils from NSCLC patients showed significantly lower killing activity against A549, SKOV-3 and SKBR cells than those derived from healthy donors (Fig. 4B, p-value < 0.05). These findings suggest that NSCLC patients' neutrophils may have an overall decreased cytotoxic potential and antitumor activity compared to those from healthy donors.

Irradiation of neutrophils does not impact neutrophil cancer cell killing activity

Since neutrophils from cancer patients showed reduced cancer killing activity as compared to neutrophils from subgroups of healthy individuals, it is possible that neutrophil adoptive transfer therapy may be an effective cancer immunotherapy. In fact, neutrophil transfusion has been approved by the FDA to treat septic neonates, neutropenic patients or patients with granulocyte dysfunction. However, one concern for neutrophil transfusion is that it may cause non-desirable adverse effects, such as graft-vs.-host (GVHD) disease. Previous studies have shown that irradiation of granulocytes may reduce such adverse side effects. To investigate whether irradiation would impact neutrophil cancer cell killing activity, neutrophils from healthy donors were treated with or without irradiation (2500 rads). As shown in Figure 5, both irradiated and non-irradiated neutrophils killed Capan-1 cancer cells effectively, as well as selectively, as cytotoxicity was not detected against non-transformed, breast epithelial cells.
These data suggest that irradiation does not negatively impact PMN cancer cell killing activity.

**Discussion**

PMNs are the most abundant circulating innate immune cells and play a critical role in immune defense against infection. However, the role of neutrophils in tumor suppression has not yet been well defined. Some early studies indicated that PMNs have the potential to kill tumor cells *in vitro.*14-17 Additionally, we have found that combined β-glucan and antitumor mAb therapy primes and activates PMN to elicit potent killing activity against iC3b-opsonized tumor cells.9,18,19 The close association of the phenomena of spontaneous tumor regression in conjunction with naturally occurring bacterial infection implicates the innate immune system in cancer immunity. Moreover, humans with a genetic defect in PMN myeloperoxidase function exhibit an elevated rate of malignant disease.20

Recent studies have shown that the action of PMN in cancer immunity is highly complex. PMNs may be subverted by transforming growth factor β (TGFβ) secreted by cells in the tumor microenvironment to a tumor supportive role,21 much like macrophages are conscripted. Although chronic inflammation has been linked to tumor initiation and progression,22 converting chronic inflammation toward an acute inflammatory response may shift some inflammatory cells, such as PMNs, into efficient anticancer agents.23 Thus, PMNs may be a double-edged sword that can exhibit both cancer promoting and inhibitory properties. A comprehensive understanding of neutrophil functionality in cancer could lead to strategies that deliberately induce “spontaneous remission.” Indeed, PMNs have been observed to exhibit a powerful antitumor effect *in vivo* under certain circumstances.24

In the current study, we demonstrate that PMNs from some healthy donors are highly effective killers of certain transformed cells, exhibiting ~70% cytotoxicity at 10:1 effector-to-target cell ratio. Five human cancer cell lines, including cells derived from mammary carcinoma (SKBR-3), non-small cell lung carcinoma (A549), cervical carcinoma (Hela), ovarian carcinoma (SKOV-3), and pancreatic carcinoma (Capan-1) were used as target cells, each of which we found to be susceptible to killing by healthy donor-derived PMNs. Importantly, this PMN-mediated killing activity is specific to tumor cells since the PMNs displayed little or no cytotoxicity against non-transformed breast epithelial cells or primary epithelial cells.
A particularly salient finding was that although some individuals harbored highly active PMNs, others possessed PMNs that exhibited virtually no anticancer activity at all. These properties appeared to be stable over at least a year in our donor population and did not exhibit any obvious relation to age (data not shown). These results imply that PMNs are an essential part of immunosurveillance critical in protecting the host from cancer development and disease progression. These PMNs may serve as safeguards eliminating cancer cells at a very early stage of tumor initiation and neoplastic progression. It would be of considerable interest to perform long term follow up studies on our healthy donor pool to determine if the degree of PMN antitumor activity inversely correlates with long-term cancer risk. In contrast, to healthy donors, PMNs from cancer patients had significantly lower average cancer killing activity. This observation suggests that either established cancers cause a state of neutrophil anergy, or that individuals with defective PMN-mediated malignant cell killing activity may have increased risk of developing cancer in the first place. It is worth noting that many other factors could contribute to the lower anticancer killing activity displayed by PMNs derived from cancer patients. For example, female smokers have significantly decreased PMN function; thus cancer patients who are smokers may have overall decreased PMN cancer killing activity that may not be directly related to either cancer risk or tumor-mediated PMN reprogramming.

The ability of PMNs to kill cells is very specific. Non-transformed cells were largely ignored by the PMNs. However, hyper-activation of certain oncogenic signaling pathways, such as the MAPK pathway or the p38 pathway, demarcates cells for killing by PMNs. Thus, certain cancers exhibiting a particular oncogene profile may be especially vulnerable to treatments that enhance or restore PMN function in cancer patients, such as granulocyte macrophage colony-stimulating factor (GM-CSF, also known as colony stimulating factor 2) or colony stimulating factor 3 (CSF3, also known as G-CSF). Previous small-scale clinical trials using GM-CSF as a single agent have reported low frequency complete remission. In addition, antitumor effects were linked to the prolonged use of G-CSF, which stimulates an intense and sustained neutrophilia with massive peritumoral PMN infiltration. Conceptually, these frequencies might be increased by enriching the subject population for those individuals with the right molecular subtype of cancer. They might also be enhanced by the addition of mTOR inhibitors such as rapamycin, which seemed to enhance the vulnerability of cancer cells to PMN attack.

The molecular mechanism by which neutrophils killed transformed cells remains poorly characterized but our findings suggest that PMN-mediated anticancer cytotoxicity requires catalase function, implicating H2O2 dependency. We also found direct contact was required as separating the tumor cells from the PMN with transwells largely abrogated the effect (data not shown).

Intriguingly, a significant number of individuals (5–25%) of African decent exhibit a physiological condition called Benign Ethnic Neutropenia (BEN). Although this effect is sub-clinical, some of these individuals have a PMN count which is naturally so low that they may be refused participation in clinical trials on the grounds that they are immunocompromised. Thus, the well known, but poorly understood, racial disparity for cancer in African American populations may, in part, reflect a PMN-based condition. Developing mechanisms for enhancing PMN function may be particularly important for malignant disease prevention in these populations.
populations. It is conceivable that adoptive transfer of PMNs from pre-screened healthy donors could be used as an effective cancer immunotherapy in these, and other patients naturally deficient in functional neutrophils.

**Materials and Methods**

**Human subjects**

Healthy individuals and human NSCLC patients were recruited at the James Graham Brown Cancer Center, University of Louisville. The study was approved by the Institutional Ethical Board and blood samples were obtained upon written informed consent and in accordance with the Declaration of Helsinki. Patients were not undergoing chemotherapy or radiation therapy at the time the blood samples were drawn.

**Plasmids and tissue culture**

TC21 (GenBank: AF493924.1), DBL (GenBank: AB085901.1), activated RhoA (GenBank: AF498970.1), activated Rac1 (GenBank: AB451238.1) and activated H-Ras (GenBank: AF493916.1) oncogenes were cloned into the pZIP-Neo2 vector and have been described previously.30-32 In brief, 1 μg of plasmid was transfected into the immortalized human breast epithelial cell line MCF-10A (American Type Culture Collection, VA) using Lipofectamine 2000 (Invitrogen, Carlsbad CA).33 Stable cell lines were obtained by selection of the transfectants in 500 μg/mL G418 (Invitrogen, Carlsbad CA). Cancer cell lines SKOV-3, Hela, A549, SKBR, and Capan-1 were obtained from the ATCC. Cancer cells were cultured in RPMI1640 or Eagle’s minimal essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS). MCF-10A cells were grown in 50/50 Dulbecco’s modified Eagle medium (DMEM)/F12 medium with 5% horse serum supplemented with epidermal growth factor, insulin and hydrocortisone, as previously described.33 Primary normal human epithelial cells were kindly provided by David Scott, University of Louisville School of Dentistry.

**Isolation of human neutrophils**

Human PMN were isolated from human peripheral blood using 2-step Ficoll/Hypaque density gradient centrifugation, as previously described.34 In brief, peripheral blood was mixed with an equal volume of 3% Dextran T-500 in PBS to sediment at room temperature for 30 min. The leukocyte-rich plasma above the sedimented red blood cells was collected and under-layered with 10 mL of 1.077 g/mL density of Ficoll-Hypaque solution (GE Healthcare, Piscataway, NJ). In addition, a second more dense layer of 1.105 g/mL Ficoll-Hypaque was added below the 1.077 g/mL layer. The tubes were centrifuged accordingly and the lower white layer occurring at the interface between the 1.077 and 1.105 g/mL Ficoll-Hypaque containing >95% neutrophils were harvested. Purity was assessed by flow cytometry with Gr-1 mAb staining (Biolegend, CA).
Neutrophil-mediated \textit{in vitro} cytotoxicity assay

Neutrophils from healthy individuals or NSCLC patients were isolated for cytotoxicity assay, as previously described.\textsuperscript{9} Cytotoxicity of tumor cells by human neutrophils was analyzed \textit{in vitro} using the RT-CES system (Acea Biosciences, Inc.; San Diego, CA), a real-time measure of the impedance of electrical current by viable target cells adhered to a conductor on the bottom of wells in a 16 well plate, according to manufacturer’s instruction.\textsuperscript{35} Briefly, 5 \times 10^3 tumor cells including Hela, SKOV-3, Capan-1, and A549 were placed into the wells of the Acea Biosciences 16 well plates for 24 h. Human neutrophils from healthy individuals or NSCLC patients were added to achieve effector-to-target (E:T) cell ratios of 10:1, 5:1, and 3:1. Cells were incubated at 37°C in a humidified 5% CO\textsubscript{2} incubator for 16 h. Cytotoxicity was calculated by measuring the relative decrease in current impedance among wells containing target cells and either neutrophils, or media only, as described previously.\textsuperscript{9,35} The percent cytotoxicity was calculated using the formula: cell index\textsubscript{medium} - cell index\textsubscript{neutrophil}/cell index\textsubscript{medium} \times 100\%. In some experiments, neutrophils were pre-treated with 5000 U/mL catalase or different inhibitors (Sigma) and then added into the culture. Inhibitors included: LY294002 (1 \mu M), SB203580 (0.5 \mu M), PD98059 (2 \mu M), rapamycin (0.1 nM), 50 \mu M AG490, 100 \mu M apocyanin, and 10 \mu M diphenylene iodonium. All experiments, with the exception of those performed for Figure 4, were repeated at least 3 times with similar results achieved. For some experiments, neutrophils were irradiated (2500 rads, GammaCell 40, Nordion, Toronto, ON) and then cytotoxicity was performed.

Nitroblue tetrazolium (NBT) assay

The production of superoxide anion in human neutrophils was determined by nitroblue tetrazolium (NBT) assay, as previously described.\textsuperscript{36} Briefly, neutrophils from healthy individuals or NSCLC patients were stimulated with or without 5 ng/mL phorbol myristate acetate (PMA) for 1 h after which NBT solution was added into wells and incubated for an additional 1 h. Media was removed and a solution of sodium hydroxide and dimethyl sulfoxide was added into each well. The plates were read in a plate reader set at 540 nm.

Statistical analysis

All quantitative data are shown as mean \pm SEM unless otherwise indicated. One-way ANOVA for multiple groups and 2-tailed, unpaired Student’s \textit{t} test for 2 groups were used for statistical analysis. Statistical analysis was performed with GraphPad Prism software and a \textit{p}-value < 0.05 was considered significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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