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Targeting FcαRI on Polymorphonuclear Cells Induces Tumor Cell Killing through Autophagy

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Neutrophils are the most abundant circulating FcR-expressing WBCs with potent cytotoxic ability. Currently, they are recognized as promising effector cells for Ab-mediated immunotherapy of cancer, because their capacity to kill tumor cells is greatly enhanced by tumor Ag-specific mAbs. The FcαRI represents the most potent FcR on neutrophils for induction of Ab-mediated tumor cell killing. However, the mechanisms of cell death that are induced are poorly understood. Because these mechanisms can be used for modulation of anticancer treatment, we investigated the mechanism of cell death induced by neutrophil-mediated Ab-dependent killing via FcαRI. Human mammary carcinoma cells were efficiently killed when incubated with human neutrophils and tumor-specific FcαRI bispecific or IgA Abs. Interestingly, we observed characteristics of autophagy such as autophagic structures by electron microscopy and LC3B+ autophagosomes in different human epithelial carcinoma cells, which resulted in tumor cell death. To a lesser extent, necrotic features, such as cellular membrane breakdown and spillage of intracellular content, were found. By contrast, apoptotic features including fragmented nuclei, Annexin V-positivity, and presence of cleaved caspase-3 were not observed. These findings indicate that neutrophils mainly facilitate autophagy to induce tumor cell death rather than the more commonly recognized apoptotic cell death mechanisms induced by NK cells or cytotoxic T cells. This knowledge not only reveals the type of tumor cell death induced in neutrophil-mediated, Ab-dependent cellular cytotoxicity, but importantly opens up additional perspectives for modulation of anticancer therapy in, for example, apoptosis-resistant tumor cells. The Journal of Immunology, 2011, 187: 726–732.

Monoclonal Abs are currently considered key therapeutic drugs for the treatment of cancer (1). They can be designed to specifically target tumor-associated Ags (TAA) and initiate several effector mechanisms, such as Ab-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity, and phagocytosis, which result in elimination of tumor cells. In addition, mAb binding to TAA can initiate signaling in the target cell, leading to apoptosis (2). Nevertheless, both in vitro and in vivo studies demonstrated that ADCC is a pre-

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Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; ADCC, Ab-dependent cellular cytotoxicity; BsAb, bispecific Ab; CC3, cleaved caspase-3; EGFR, epidermal growth factor receptor; EM, electron microscopy; HER-2/new, human epidermal growth factor receptor 2; MeOH, methanol; PMN, polymorphonuclear cell; RT, room temperature; TAA, tumor-associated Ag.

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cells as effector cells in ADCC. Second, necrosis is the result of cellular stress (e.g., a toxic environment or physical damage) up to a level incompatible with cell survival (18). Third, autophagy has recently been identified as an alternative, nonapoptotic route of cell death (19–21). Autophagy was first described to play a critical role in cellular survival by preserving cell energy. Nevertheless, it is now clear that under excessive stress conditions or when cells are resistant for apoptosis induction, autophagy becomes an important alternative cellular suicide pathway (22).

Previously, PMN-mediated ADCC has been investigated using standard chromium release assays (23). With this method, adherent tumor cells were trypsinized, labeled for several hours with chromium, and subsequently used for an “in suspension” ADCC assay. However, this method influences cell death, as keeping adherent cells in suspension might induce anokis (apoptosis triggered by cell detachment) (24). In this study, we therefore investigated the underlying type of tumor cell death of adherent tumor cells induced by PMNs. By coculturing adherent carcinoma cells and PMNs with anti-tumor Ag-specific FcγR IgA or IgG Abs, we observed early-onset tumor cell kill, which was independent of apoptosis but coincides primarily with characteristics of autophagy. These findings provide new insight on cell death and open up new possibilities of using FcγRi expressing PMNs for Ab therapeutics, not only for apoptosis-resistant tumor cells, and also offer opportunities to maximize tumor cell death by using different effector cell types for induction of distinct cell death pathways.

Materials and Methods

Cell lines

The human breast carcinoma cell line SK-BR-3, which overexpresses the TAA HER-2/new and the human epithelial carcinoma A-431 (epidermal growth factor receptor [EGFR] as TAA), were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 medium (SK-BR-3) or DMEM (A431; Life Technologies, Paisley, Scotland, UK) supplemented with 10% heat-inactivated FCS (Integro, Dieren, The Netherlands) and antibiotics.

Isolation of effector cells

PMNs were isolated from heparin anticoagulated peripheral blood drawn from healthy volunteers by standard Lymphoprep isolation (Axis-Shield, Rodelolka, Oslo, Norway) according to manufacturer’s protocol. Erythrocytes were removed by hypotonic lysis buffer (155 mM NH4Cl2, 10 mM KHCO3, and 0.1 mM EDTA). Purity of PMNs was determined by cytospin preparation and always exceeded 95%. Cell viability of PMNs exceeded 98% as assessed by trypan blue staining. NK cells were isolated and stimulated with IL-15 as described in Verhoeven et al. (25). Studies were approved by the Medical Ethical Committee of VU University Medical Center (The Netherlands) in accordance with the Declaration of Helsinki. All donors gave informed consent.

mAbs and BsAbs

mAb A77 (mlgG1 anti-FcαRI) and 520C9 (mlgG1 anti–HER-2/neu) were produced from hybridomas (Medarex, Bloomsbury, NJ). FcRιxHER-2/new BsAb (A77x520C9) was produced by chemically cross-linking F(ab’) fragments of A77 with F(ab’) fragments of 520C9, as described in Fanger et al. (26). Anti-EpCAM IgA was kindly provided by Crucell (Leiden, The Netherlands), and anti-EGFR IgA Ab was produced as described in Beyer et al. (27). Anti-cleaved caspase-3 (anti–CC3) and LC3B Abs were purchased by Cell Signaling Technology (Danvers, MA).

ADCC

Tumor cells were seeded in 96-well plate or glass coverslide. The next day, PMNs were added in an E:T ratio of 10:1 or 30:1 in the presence or absence of indicated (bispecific) Abs at a final concentration of 1 or 2 μg/ml at 37°C in a humified atmosphere containing 5% CO2. Supernatants, containing detached tumor cells and PMNs, were used for cytospins to avoid contamination with viable tumor cells. Slides and cytospins were stained with Mayer’s H&E (Klinipath, Duiven, The Netherlands), after which they were dehydrated in ethanol and embedded in Entellan (Merck, Haarlem, The Netherlands). The number of viable cells remaining in 96-well plates was determined with standard MTT assays as described by Heuff et al. (28). The apoptosis inhibitor Z-VD-fmk was purchased from Biocompare (San Francisco, CA). GFP–LC3-RFP cDNA was stably transfected in SK-BR-3 cells with FuGENE 6 transfection reagent according to manufacturer’s recommendations.

CC3 and LC3B stainings

To determine CC3 or LC3B staining on cytopsins, cells were fixed with 4% paraformaldehyde (pH 7.4) for 15 min and further fixed and permeabilized with ice-cold (−20°C) 100% methanol (MeOH) for 5–10 min. Cells were blocked with 10% normal swine serum and further permeabilized for 1 h at room temperature (RT) in PBS containing 1% BSA, and 0.1% saponin. For CC3 stainings, cells were incubated overnight at 4°C with anti–CC3 mAb and subsequently incubated with biotinylated swine anti-rat Abs for 60 min at RT. Cells were then incubated with streptavidin–AP for 60 min at RT, and CC3 staining was visualized by adding AP-substrate (SIGMAFAST p-Nitrophenyl phosphate [pNPP]; Sigma-Aldrich, St. Louis, MO). Cells were counterstained with H&E to enable visualization of their nuclei before they were air-dried and mounted (original magnification ×40). For detection of autophagy, anti–LC3B mAb were used according to the manufacturers’ protocol. Fluorescence was visualized using an LM LEICA DM 6000 microscope, original magnification ×40 (Leica Microsystems, Wetzlar, Germany).

Electron microscopy

Cells used for electron microscopy (EM) analysis were fixed in 2.5% glutaraldehyde, postfixed with 1% osmium tetroxide, dehydrated in ethanol, infiltrated with propylene oxide, and embedded in Agar 100 Resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a transmission electron microscope (Philips CM 100 BioTwin; Philips, Eindhoven, The Netherlands).

Statistical analysis

Results were expressed as means ± SD. Statistical analysis was performed with paired Student t test (two groups) or ANOVA (more than two groups). The p values <0.05 were considered statistically significant.

Results

SK-BR-3 tumor cell death induced by PMNs and FcαRI BsAb

Because it has been described that adherent cells, which are kept in suspension, can go into anokis (apoptosis triggered by cell detachment) (24), we developed a novel plate-bound ADCC assay (described in Materials and Methods). Coculturing adherent SK-BR-3 breast cancer cells with PMNs for 3 h in the presence of different concentrations of FcRιxHER-2/new BsAb (520C9xA77), parental IgGxHER-2/new mAb (520C9), or without Ab resulted in specific FcγRI-induced detachment of SK-BR-3 cells (Fig. 1A). Moreover, even with a low E:T ratio, PMNs were able to detach tumor cells in the presence of FcγRI BsAb. Minimal detachment of SK-BR-3 cells was measured using either parental IgG mAb or in the absence of Ab. Detached cells were reseeded to establish whether the detached SK-BR-3 cells had indeed been killed by PMNs in the presence of FcγRI BsAb. After 2 wk of culturing, 75% reduction in viable tumor cells was observed.
when compared with freshly seeded SK-BR-3 cells (Fig. 1B), indicating that the majority of detached SK-BR-3 cells treated with BsAb and PMNs were nonviable. Of note, determining SK-BR-3 tumor cell death with classical chromium release assays using FcαRI BsAb and PMNs demonstrated similar amounts of cell death (60–80%) compared with this plate-bound ADCC assay (15, 29). Thus, significant tumor cell death can be induced with PMNs and FcαRI BsAb, and can be quantified using this plate-bound ADCC assay.

FcαRI BsAb-induced cell death of SK-BR-3 cells is independent of apoptosis

Because NK cell-mediated ADCC induces apoptosis in tumor cells (30), we investigated whether apoptosis was also the cell death mechanism induced in tumor cells when neutrophils were used as effector cells. Characteristics of apoptotic cells are intact plasma membranes, nuclear fragmentation, presence of apoptotic bodies, and positive staining for CC3. Staurosporine, which is a potent inducer of apoptosis, was used as a positive control (31). In contrast with staurosporine-treated SK-BR-3 cells, neither positive CC3 staining nor fragmented nuclei nor apoptotic bodies were observed in SK-BR-3 cells that had been incubated with PMNs and FcαRI BsAb (Fig. 2A). Furthermore, neither morphology changes nor changes in levels of tumor cell death were observed after incubation of SK-BR-3 cells with PMNs and FcαRI BsAb in the presence of Z-VAD-fmk, which is a known inhibitor of apoptosis activity (Fig. 2B, 2C). Z-VAD-fmk did inhibit induction of apoptosis in cells treated with staurosporine. Thus, PMN-mediated ADCC using FcαRI BsAb does not involve induction of apoptosis.

Characteristics of SK-BR-3 cells killed via apoptosis, autophagy, or necrosis

To investigate which other type of SK-BR-3 cell death is induced during PMN-mediated ADCC, we examined characteristics of the three major forms of cell death and compared this with cell death observed in SK-BR-3 cells cocultured with PMNs and FcαRI BsAb. Cells cultured in medium only were used as viable cells (Fig. 3A). Staurosporine-treated cells were used for induction of apoptosis (Fig. 3B), whereas autophagy (Fig. 3C) or necrosis (Fig. 3D) were induced by rapamycin (22) or MeOH treatment, respectively (32). Detached nonviable cells in supernatants were used to assess different cell death morphology, compared with viable cells by H&E-stained cytospins and EM, as the latter technique provides more detailed cell morphology and is considered the “gold standard” for distinguishing apoptosis from autophagy and necrosis (17, 18). To visualize the process of cell death induction in time, we used fluorescence lifetime microscopy for 5 h. Annexin V-FITC (a marker for apoptosis) and 7-AAD, which binds DNA and is a marker for general cell death, were present during recording (Supplemental Video 1; fluorescence lifetime microscopy movies). Whereas viable cells (Fig. 3A) showed an intact cell membrane and nucleus (N) with nucleoli,
form fragmented apoptotic nuclei (Na) were visible in the staurosporine-treated cells (Fig. 3B). Furthermore, autophagic features like intracellular vacuolization and double-membrane structures were observed in rapamycin-treated cells (indicated by Av, Fig. 3C), whereas necrotic SK-BR-3 cells clearly showed disruption of the cell membrane and spillage of cellular contents (Fig. 3D). Of note, changes in nuclear morphology are exhibited in both autophagic and necrotic cells. However, no apoptotic features like nuclear condensation and fragmentation were observed as demonstrated in staurosporine-treated cells (Fig. 3B) and as described by Amaravadi and Thompson (18). With fluorescence lifetime microscopy, neither morphology changes nor Annexin V and 7-AAD fluorescence were observed in cells that were kept in medium after 5 h (Fig. 3A). However, appearance of apoptotic bodies and shrinkage of cells was observed in staurosporine-treated cells (Fig. 3B). In addition, over time, staurosporine-treated cells became positive for both Annexin V and 7-AAD, which reflects cells undergoing apoptosis. On the contrary, rapamycin-treated cells (inducing autophagy) demonstrated vacuolization in the cytoplasm and became positive for 7-AAD (Fig. 3C). Similarly, necrotic cells induced by MeOH treatment became 7-AAD+, and additionally showed breakages of cellular membrane, as well as leakage of cellular content (Fig. 3D). Staurosporine, rapamycin, and MeOH treatment resulted in detachment of tumor cells, indicative for cell death (see also Fig. 1). Thus, the earlier described results clearly demonstrated distinct differences between SK-BR-3 cells dying of apoptosis, autophagy, and necrosis. These conditions and techniques were subsequently used to determine PMN-induced tumor cell death.

PMN-induced cell death of SK-BR-3 cells shows autophagic and necrotic features

Using PMNs and FcαRI BsAbs in a plate-bound ADCC assay induced tumor cell kill and revealed morphological changes in SK-BR-3 cells compared with the control (PMNs without BsAb; Fig. 4A, three representative photomicrographs are shown). SK-BR-3 cells were counted and subdivided in cells without visible changes (36%), apoptotic features (1%; as apoptotic nuclei can be distinguished by normal microscopy), and cells with non-apoptotic feature changes (63%; Fig. 4A, right graph). EM data of these cells demonstrated a heterogeneous phenotype (Fig. 4B, four representative photomicrographs are shown). Necrotic features like disruption of the cell membrane with leakage of cellular contents were observed (Fig. 4B, indicated by arrow in upper left panel). However, the majority of cells demonstrated characteristics of autophagy such as vacuolization and numerous double-membrane-bound vesicles (Av) that frequently contained electron-dense materials of cytoplasmic fragments and organelles (Fig. 4B).
4B with insets). Compared with viable cells, changes in nuclear morphology were observed, although neither fragmented nor pyknotic nuclei were observed (for comparison, see apoptotic cell in Fig. 3B). Moreover, SK-BR-3 cells incubated with PMNs and BsAb followed by fluorescence lifetime imaging demonstrated cell death, as indicated by 7-AAD+ cells, but no Annexin V+ tumor cells (Fig. 4C, Supplemental Video 2). Of note, an enhanced number of apoptotic PMNs ( Annexin V+ PMNs) was observed in the BsAb samples (33, 34). For comparison, NK cell-mediated lysis of these tumor cells was also investigated by fluorescence lifetime imaging. As expected, NK cells induced apoptosis (30) in SK-BR-3 cells (Annexin V+ SK-BR-3 cells and Annexin V+ NK cells) in combination with anti-EGFR IgA Abs (Fig. 4C). However, NK cells did not induce apoptosis in SK-BR-3 cells incubated with PMNs and BsAb (Fig. 4C), indicating that PMNs induce a different type of cell death in SK-BR-3 cells compared with NK cell-mediated apoptosis. Another distinct hallmark of autophagy is the translocation of LC3B from the cytosol to autophagosomes (21, 35). This inhibitor prevents the degradation of LC3B in autophagosomes and inhibits fusion with lysosomes to form autolysosomes, which are both required for autophagic cell death (21). In the presence of this inhibitor, SK-BR-3 cells were rescued from cell death induced by either PMNs and BsAb or rapamycin. To demonstrate that induction of autophagy was not dependent on TAA or tumor cells, we also performed ADCC experiments with anti-EpCAM IgA Abs and SK-BR-3 cells (Supplemental Fig. 2). Also in these settings, autophagy was induced, demonstrated by GFP+ and RFP+ vesicles (Supplemental Fig. 2B) or by endogenous LC3B stainings (Supplemental Fig. 2D). These results together imply that FcγRI-mediated ADCC by PMNs induced cell death, which primarily coincided with autophagic characteristics but not apoptotic characteristics and was independent of TAA or tumor cell type.

Discussion

FcyR-bearing cells, such as NK cells, monocytes, and macrophages, are important for induction of cytolytic activity in vivo to induce Ab-mediated tumor regression (36–38). More recently, PMNs have been documented as promising candidate effector cells in ADCC, but the mechanisms of target cell death remained poorly investigated (12).

In this study, we observed early-onset cell death of adherent mammary carcinoma SK-BR-3 cells when incubated with PMNs as effector cells in the presence of FcγRI BsAb. Less effective ADCC was observed via FcγRs (Fig. 1A) (15, 29, 39). We recently demonstrated that targeting FcγRI, but not FcγRs, induces...
an autocrine PMN migration loop through release of leukotriene B₄, leading to recruitment of PMNs (40). Accordingly, we also observed recruitment of PMNs toward the tumor colonies in our ADCC experiments only in the presence of FcγRI BșAb. Thus, increasing numbers of effector cells may underlie the enhanced ADCC capabilities demonstrated when using FcγRI as target FcR on PMNs. To investigate type of tumor cell death induced by PMNs, we mainly used SK-BR-3 tumor cells, FcγRI, and HER2/neu BșAb, because this combination is efficient in inducing tumor killing. This resulted in tumor cell death, which was independent of apoptosis but primarily accompanied by characteristics of autophagy. Because PMNs bind with high affinity to tumor cells in the presence of BșAbs, and PMN mainly die through apoptosis, microscopy was used to visualize cell death, as this allowed discrimination between either dying PMNs or tumor cells. EM of SK-BR-3 cells incubated with PMNs and BșAb revealed double-membrane vesicle formation. Moreover, expression of the autophagy marker LC3B, as well as inhibition of cell death in the presence of ammonium chloride (inhibiting lysosomal proteolysis and fusion between autophagosomes and lysosomes, essential for autophagic cell death), indicate the presence of autophagolysomes, and thus supports autophagic cell death mechanisms (21). Unfortunately, autophagy inhibitors like 3-MA (PI3K inhibitor), SB600013 (JNK inhibitor), or bafilomycin A (inhibiting fusion between autophagosomes and lysosomes), could not be used in our ADCC because these inhibitors also affected PMN activation. In addition, evidence of necrotic cell death was observed in light microscopy and EM. However, whether necrosis is a truly separate cell death mechanism or coincides with autophagic cell death mechanism could not be discriminated from our experiments, because cells undergoing necrosis often show characteristics of autophagy (17). Nonapoptotic cell death mechanisms were further confirmed using lifetime fluorescence microscopy demonstrating 7-AAD⁺ cells. Thus, we demonstrate for the first time, to our knowledge, that primarily autophagy and, to a lesser extent, necrosis contributes to early-onset cell death in FcγRI-mediated ADCC, with PMNs as effector cells, independently of apoptosis. Importantly, we could also demonstrate that induction of autophagy by PMNs is neither dependent on tumor cell line nor TAA by using other tumor cells (A431) or anti-tumor–specific IgA Abs (EpCAM and EGFR).

Induction of apoptosis in “end-stage” killing of HER-2/neu-expressing tumor cells by PMNs (after 20 h) was described, although the exact mechanism of this apoptosis pathway was not identified (41). An explanation for the different observations might be that SK-BR-3 cells were used in suspension during the 20-h assay and detachment of cells induces anoikis (apoptosis triggered by cell detachment) (24). A nonapoptotic early-onset tumor cell death, induced by PMNs, is supported by data of Metelitsa et al. (42). Although in this study tumor cell death mechanism was not thoroughly investigated, EM photomicrographs of ADCC samples clearly demonstrated absence of apoptotic features in nonviable neuroblastoma cells. Of note, autophagolysosomes could not be detected because of low magnification of the photomicrographs.

Apoptosis is induced via release of cytotoxic granules (containing perforin, granulysin, and granzymes) or via activation of the TNF family of death receptors (TNFR, Fas, and TRAILR) (37). Although it was shown that INF-α-stimulated PMNs can release a soluble form of TRAIL (TRAIL/APO2L) (43), release of perforin and granzymes by PMNs is controversial (44, 45). This suggests that PMNs facilitate other cell death mechanisms for tumor cell eradication. Thus, by activating different cell types during ADCC, specific cell death mechanisms can be induced. This will give more insight in mechanisms of anticancer therapies and can be used to further optimize clinical outcome (46). Especially because, in many patients, cancer cells harbor mutations that result in resistance to apoptosis, which emphasizes the need for new approaches that exploit nonapoptotic cell death modes for anticancer therapies (47). Currently, several clinical trials are testing strategies of eliminating tumor cells by inducing autophagy as described for human T lymphocyte leukemia treated with arsenic trioxide or treatment of chronic myeloid leukemia with imatinib (46). Also, rapamycin derives demonstrate promising results as an anticancer therapy (48).

In summary, although autophagy is well embedded as a cell survival mechanism, we and others demonstrated a role for autophagy as cell death mechanism and underline the potential use of
autophagy for cancer treatment. This can be induced by targeting FcγRI on PMNs either with BsAb or IgA Abs, and therefore represents a promising addition to the so far IgG-dominated, Ab-mediated anticancer treatments.

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