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NEUTROPHIL-TUMOR CELL PHAGOCYTOSIS (CANNIBALISM) IN HUMAN TUMORS: AN UPDATE AND LITERATURE REVIEW

R.A. Caruso, F. Fedele, G. Finocchiaro, G. Arena, A. Venuti*
Department of Human Pathology, University of Messina, Messina, Italy

The recognition and removal of apoptotic cells by tissue macrophages and nonprofessional phagocytes, in a process called efferocytosis, is critical for development, tissue homeostasis and resolution of inflammation. Apoptotic bodies arising in tumor tissue are ingested by viable neoplastic cells and by resident macrophages. We described tumor cell phagocytosis of apoptotic neutrophils in human gastric carcinomas. This phenomenon is analogous to neutrophil efferocytosis performed by macrophages and by nonprofessional phagocytes during inflammatory reaction but is distinct by other types of cell-in-cell phenomena including emperipolesis and entosis both cytologically and biologically. In this review, we discussed them in their ultrastructural morphology, physiological roles, and clinicopathologic implications. This article is part of a Special Issue entitled "Apoptosis: Four Decades Later".
Key words: **tumor cell phagocytosis, cannibalism, electron microscopy.**

INTRODUCTION

The expression "apoptosis" has been coined by Kerr *et al*. [1] to describe the phenomenon as a mode of cell death morphologically separate from coagulative necrosis. Apoptosis is characterized by the sequence of nuclear shrinkage (pyknosis), chromatin margination, nuclear fragmentation (karyorhexis), and engulfment by neighboring cells. All of these changes occur before plasma membrane integrity is lost [2]. Electron microscopy permits the visualization of fine ultrastructural modifications that accompany cell death, including gaps in the plasma and/or in the mitochondrial outer membrane [3], mitochondrial swelling [4], and the first phases of chromatin condensation (which only later become visible by light microscopy) [5]. Phagocytosis of apoptotic cells, also known as *efferocytosis*, is performed by both professional phagocytes (such as macrophages and dendritic cells) and by nonprofessional "neighboring" phagocytes (such as epithelial cells, endothelial cells, and fibroblasts) [6]. For example, viable epithelial cells recognize and engulf their apoptotic neighbours in normal epithelia *in vivo* [7], while the glomerular mesangial cell ingests both leucocytes and adjacent apoptotic glomerular cells during the resolution of renal inflammation [8]. More recently, mouse mammary gland epithelial cells have been reported to ingest apoptotic alveolar cells during the process of mammary gland involution [9].

NEUTROPHIL EFFEROCYTOSIS

Neutrophils are classified as professional phagocytes, and are important in resolution and clearance of pathogens. They are known to phagocytose pathogens (including yeast and bacteria) as well as potentially hazardous substances, being a first line defence. Neutrophils are inherently short-lived cells with a half life of only ~ 6–10 h in the circulation and rapidly undergo spontaneous apoptosis [10]. Apoptotic inflammatory neutrophils are phagocytosed primarily by macrophages, provided these cells are present in adequate numbers. However, macrophages are rare at sites of acute inflammation, whereas the number of neutrophils can be extremely high [11]. Therefore, nonprofessional phagocytes and/or viable neutrophils can participate in the clearance of apoptotic neutrophils [11].

There is surprisingly little data about efferocytosis of neutrophils by epithelial cells. Sexton *et al*. [12] have demonstrated that human epithelial cells from different regions of the lung recognize and ingest apoptotic eosinophils but not apoptotic neutrophils. Thus, human airway epithelial cells might be active participants in the removal of apoptotic eosinophils and therefore play an important role in the resolution of eosinophilic inflammation in the asthmatic lung [12]. Sexton *et al*. [12] suggest that epithelial cells at different organ sites retain a selective ability to recognize and engulf only one of the two apoptotic granulocytes. The reasons for such differences are unclear but may reflect the employment of subsets of cooperating phagocytic receptors for particular apoptotic cell types [12]. We are currently examining a large number of chronic active *H. pylori* gastritis for the presence of apoptotic neutrophils within foveolar epithelium. Transmission electron micrographs revealed apoptotic neutrophils within spacious phagosomes of foveolar cells. These findings are compatible with late phase of neutrophil efferocytosis by foveolar cells and suggest their anti-inflammatory role in the removal of apoptotic neutrophils during acute foveolitis. The clinical implications of our findings in these patients are under study.

"PHYSIOLOGICAL" AND "PATHOLOGICAL" INFLAMMATION

Because uncontrolled release of toxic substances from dead neutrophils can propagate the inflammatory response leading to tissue destruction, recognition of dying inflammatory neutrophils has a critical function for the resolution of the inflammatory response [13]. It leads not only to the removal of the inflammatory cells themselves, along with anything they have in-

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*Correspondence: E-mail: rosariocaruso@tin.it

gested, but also to the generation of anti-inflammatory mediators that shut down the ongoing inflammation [14]. This condition may be defined "physiological" inflammation [14]. However, if macrophages and/or nonprofessional phagocytes fail to clear the apoptotic neutrophils, apoptotic neutrophils are left in the tissue and undergo secondary necrosis: a process characterized by uncontrolled leakage of the dying cell contents resulting in a propagated inflammatory response [13]. In this condition of "pathological" inflammation activated immune cells, primarily represented by neutrophils, macrophages, and cytotoxic T cells, play the role of aggressors that attack and destroy nearby cells, either directly through physical contact or indirectly through the release of soluble factors such as reactive oxygen and nitrogen metabolites, cytotoxic proteins, lytic enzymes, or cytokines [14, 15]. Therefore, neutrophils are potentially very cytotoxic, mainly when they lyse [16]. The harm associated to the participation of neutrophils in inflammatory situations can be considerable, as neutrophils are usually recruited in high number to inflammatory/infectious sites [16]. These features of neutrophil participation in inflammatory situations associated to infection or tissue injury make the neutrophil a dangerous cell that must be tightly controlled.

TRANSEPITHELIAL NEUTROPHIL MIGRATION

Common to the rapidly growing, multifaceted literature on mechanisms of resolution of inflammatory disease processes is the centrality of leukocyte apoptosis followed by phagocytosis of the apoptotic leukocyte [13, 17]. Apoptosis mechanisms are consistently emphasized whereas it appears that a role of transepithelial cell migration may have been overlooked. Contributing to the limited attention, another prevailing dogma has taught that the transepithelial movement of leucocytes is an injury-evoking, pathogenic component of mucosal inflammatory diseases [18]. The transepithelial leucocyte egression is required for surveillance, sentinel and defence duties to be carried out in part on the mucosal surface and, likely, also in the lumen [18]. Migration of neutrophils across the mucosa may be critical to a successful combating of mucosal infections [19]. Hence, it is reassuring that the transepithelial exit of granulocytes can occur without compromising the integrity of the mucosal epithelial barrier [20, 21]. Epithelial restitution after shedding-like epithelial damage, which does not injure the basement membrane, can be a very rapid process *in vivo* [22]. However, for a well-functioning disease-resolving process, transepithelial elimination of leucocytes would have to occur without inflicting any injury at all [22]. Occurrence of inflammatory cells such as eosinophils, neutrophils, lymphocytes, dendritic cells and mast cells in the lumen of mucosal lined hollow organs has demonstrated that transepithelial elimination of such cells is operational at disease resolution [18]. Many clinical observations and pharmacological *in vivo* evidence, accumulating in recent decades, are compellingly supportive [18]. Hence, transepithelial migration emerges as an additional mode of ridding diseased mucosal tissues of inflammatory cells. However, a resolving role of moving leucocytes into the lumen of hollow organs has limitations [23]. For example, a distinction must be made between egression of infiltrated leucocytes across mucosal epithelia where a swift further elimination of the lumen cells can be expected to occur (nasal, tracheobronchial, gut and bladder mucosae) and the bronchiolar-alveolar epithelial linings where there is a risk of harmful accumulation of lumen cells [22]. Neutrophil persistence in the airways can occur through a number of mechanisms such as impaired apoptosis, efferocytosis and mucus hypersecretion, all of which are impaired in airways disease [22]. Impairment of neutrophil clearance results in a reduced ability to respond to bacterial infection [22]. Persistent activation of airway neutrophils may result in the persistent activation of the innate immune system resulting in further airway insult [22].

EMPERIPOLESIS AND ENTOSIS

Humble *et al*. [24] coined the term "emperipolesis" in 1950s to define the heterogeneous cell-in-cell phenomena when they studied biological interaction of lymphocytes with other cells. Emperipolesis has since been found to be commonly enacted by lymphocytes in physiological and pathophysiological settings [25]. Recently, Overholtzer *et al*. [26] observed a homogeneous cell-in-cell phenomenon and named it as "entosis", an intercellular process that exhibits remarkable similarity to emperipolesis. Entosis has been observed in many cell lines at varying frequencies, including breast epithelial cells, breast carcinoma cells, and human embryonic kidney cells [27]. Internalized cells initially appear healthy and viable; some even divide while inside of the host [28]. Over a period of 20 hours, some internalized cells are able to escape, but most cells die through a specialized form of cell death that lacks hallmarks of apoptosis, as dying cells are negative for cleaved caspase-3 and do not exhibit condensed or fragmented nuclei [26, 29].

PHAGOCYTOSIS: A SHARED BEHAVIOR OF M2 MACROPHAGES AND TUMOR CELLS

Tumor cell cannibalism was described as the ability of tumor cells to cannibalize their siblings as well as cells from the immune system [30, 31]. In cytological or histological samples of human tumors it is a common finding to detect cells with one or more vacuoles, possibly containing cells under degradation, that push the nucleus to the periphery giving it the shape of a crescent moon [32]. The process of entosis is different from cannibalism in that entosis is a live cell invasion while cannibalism has no selectivity for dead cells or live cells [26–28]. Furthermore, entosis is a homogeneous cell-in-cell phenomenon while cannibalism can be either homogeneous or heterogeneous cell-in-cell structures [33, 34]. While phagocytic behaviors

have been reported for most forms of human cancer, not all cancer cells within a tumor are phagocytic. For most of the tumor described, phagocytic/cannibalistic behavior was restricted primarily to those cells that are highly invasive and metastatic [35–37].

Phagocytosis is a specialized behavior of M2 macrophages and other professional phagocytes. M2 macrophages also express high levels of lysosomal-enriched cathepsins, which facilitate the digestion of proteins ingested following phagocytosis [38, 39]. Interestingly, lysosomal cathepsins D and B are viewed as prognostic factors in cancer patients [40, 41]. A high content of these enzymes in tumors of the head and neck, breast, brain, colon, or endometrium was considered a sign for high malignancy, high metastasis, and overall poor prognosis [42]. These data suggest that numerous human cancers express multiple properties of M2 macrophages including phagocytosis and protein expression.

TUMOR CELL PHAGOCYTOSIS/ CANNIBALISM OF NEUTROPHILS

The phenomenon of neutrophil-tumor cell emperipolesis or phagocytosis (cannibalism) has been documented by the light microscopy in pleomorphic (giant) cell carcinomas of the lung, gall bladder, pancreas, and intestine [43–47]. Pleomorphic (giant) cell carcinoma is defined as a tumor lacking any identifiable glandular, squamous or other differentiation [48]. The characteristic histologic findings include marked pleomorphism, lack of cohesiveness of tumor cells, aggregates or sheets of mononucleated and multinucleated giant cells, and extensive necrosis [48]. One peculiar finding in invasive micropapillary carcinoma of ampullo-pancreatobiliary region noted by Khayyata *et al*. [49] is the presence of tumor-infiltrating neutrophils. Neutrophils were abundant and could be identified both within the carcinoma cells ("cannibalism") as well as in the stroma adjacent to the tumor cells [49]. Furthermore, the neutrophils showed striking tumoral-centric distribution, decreasing in numbers away from the tumor cells. Thus, phenomenon of neutrophil-tumor cell emperipolesis or phagocytosis (cannibalism) has been reported in pleomorphic giant cell carcinoma and in micropapillary carcinomas: two histologic types characterized by a poor prognosis.

In order to study the phenomenon of neutrophil-tumor cell emperipolesis or phagocytosis (cannibalism) in gastric tumor, we reviewed and analyzed the ultrastructural findings observed in 9 cases of advanced gastric carcinomas [30]. An intraepithelial localization of neutrophils was seen in 2 out of 9 cases (Fig. 1). They showed the characteristic equipment of discrete primary and secondary granules, glycogen and lipid bodies. Other neutrophils were present within vacuoles of adenocarcinoma cells and showed various phases of apoptotic changes. Ultrastructural signs of early apoptosis included nuclear chromatin separation into dense and electron lucent areas, rounded nuclear profiles, preservation of cytoplasmic granules, and maintenance of cell membrane integrity of neutrophils (Fig. 2). Late apoptotic morphology was characterized by cell shrinkage, tightly packed cytoplasmic granules, and uniform collapsed nucleus (Fig. 3) [30].

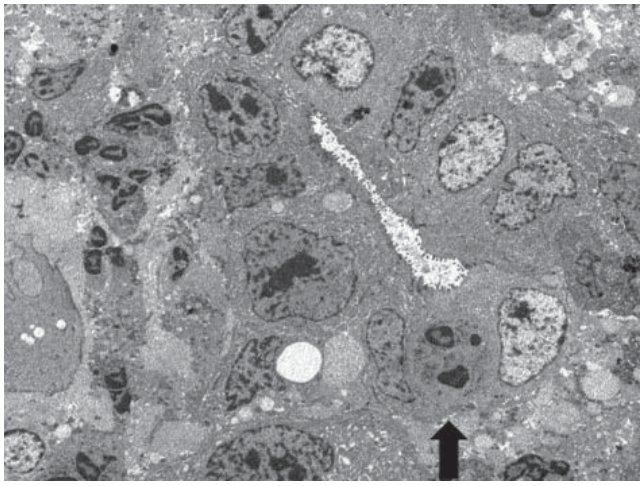

Fig. 1. Intestinal-type gastric adenocarcinoma with intraepithelial neutrophil localization (arrow) × 4 000

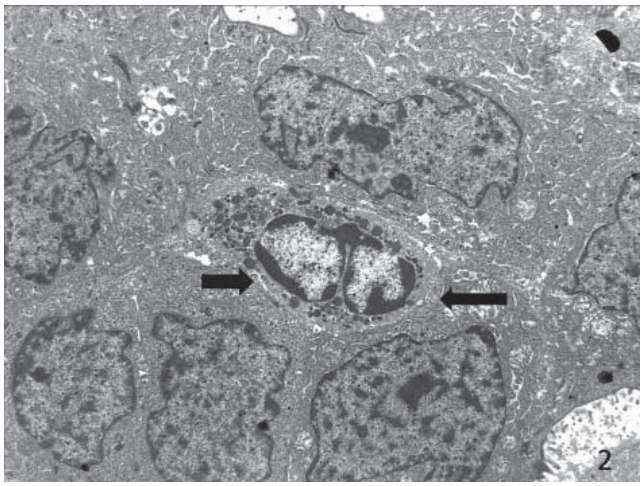

Fig. 2. Intestinal-type gastric adenocarcinoma. An intraepithelial neutrophil shows early apoptotic changes, including nuclear chromatin separation into dense and electron-lucent areas (arrows), preservation of cytoplasmic granules, and maintenance of cell membrane integrity. × 6 000

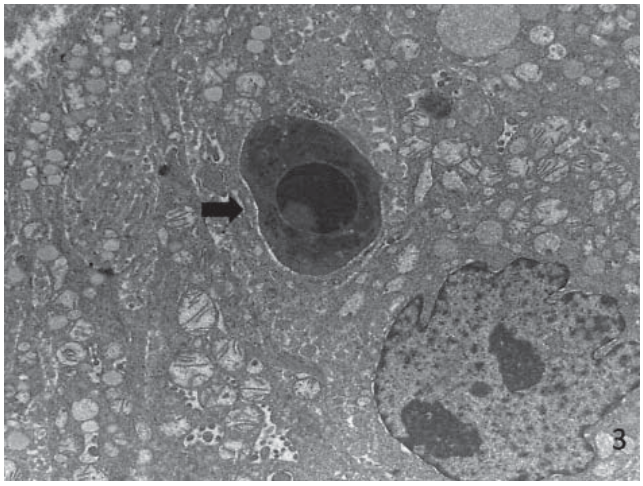

Fig. 3. Adenocarcinoma showing intraepithelial neutrophil with late apoptotic changes such as cell shrinkage, tightly packet cytoplasmic granules, and uniform collapsed nucleus (arrow). × 6 000

Secondary degeneration of apoptotic neutrophils within the phagocytic vacuoles of tumor cells included cellular swelling, electron-lucent cytoplasm,

vacuolization and indiscernible cell membrane (Fig. 4). Viable neutrophils, cell debris and apoptotic neutrophils were also observed within the lumina of gastric tumor (Fig. 4 and 5). In summary, the ultrastructural study shows the presence of apoptotic neutrophils within cytoplasmic vacuoles of adenocarcinoma cells. Therefore, the phagocytic nature (cannibalism) of this interaction was demonstrated, and the possibility of emperipolesis/entosis was excluded.

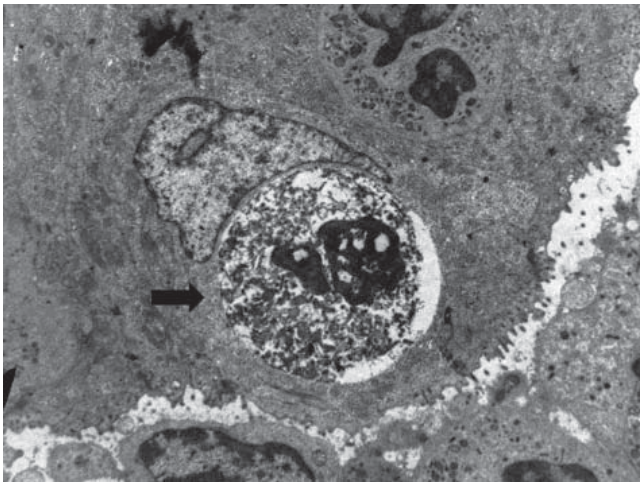

Fig. 4. Neutrophil-tumor cell phagocytosis. Late apoptotic neutrophil is contained within spacious phagosome (arrow). Note various stages of secondary degeneration in phagocytosed neutrophil including swollen cytoplasm, loss of glycogen granules, vacuolization, and indiscernible cell membrane. × 10 000

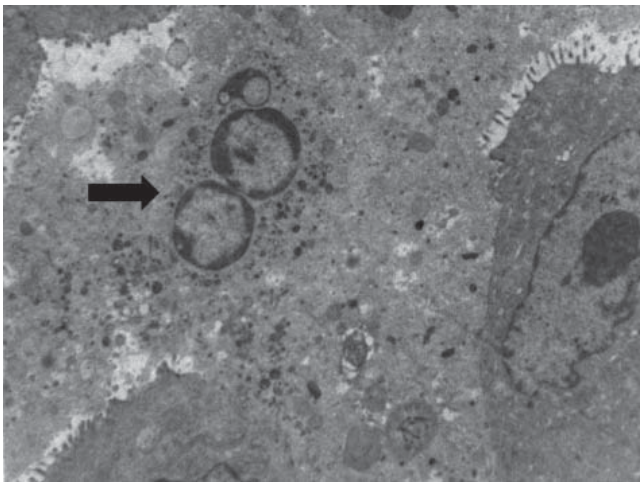

Fig. 5. Adenocarcinoma containing an apoptotic neutrophil (arrow) and cell debris. × 10 000

MALIGNANT TUMOR CELLS FEED ON INGESTED CELLS

Recently, Lugini *et al*. [35] investigated the occurrence, the *in vivo* relevance, and the underlying mechanisms of cannibalism in human melanoma. As first evidence, they observed that tumor cannibalism was clearly detectable *in vivo* in metastatic lesions of melanoma and often involved T cells, which could be found in a degraded state within tumor cells. Then, *in vitro* experiments confirmed that cannibalism of T cells was a property of metastatic melanoma cells but not of primary melanoma cells. In particular, morphologic analyses, including time-lapse cinematography and electron microscopy, revealed a sequence of events, in which metastatic melanoma cells were able to engulf and digest live autologous melanoma-specific $CD8^+$ T cells [35]. Importantly, this cannibalistic activity significantly increased metastatic melanoma cell survival, particularly under starvation condition, supporting the evidence that tumor cells may use the eating of live lymphocytes as a way to "feed" in condition of low nutrient supply [35]. The mechanism underlying cannibalism involved a complex framework, including lysosomal protease cathepsin B activity, caveolae formation, and ezrin cytoskeleton integrity and function. In conclusion, Lugini *et al*. [35] suggest that cannibalism may represent a sort of "feeding" activity aimed at sustaining survival and progression of malignant tumor cells in an unfavorable microenvironment.

HORIZONTAL TRANSFER OF DNA BY UPTAKE OF APOPTOTIC BODIES

It has been generally considered that DNA from dying cells is degraded after apoptosis and, thus, inactivated. However, recent studies have demonstrated that horizontal DNA transfer between mammalian cells can occur through the uptake of apoptotic bodies, where genes from the apoptotic cells were transferred to neighbouring cells phagocytosing the apoptotic bodies [50–54]. The regulation of this process is poorly understood. p53, via the activation of p21, blocks normal cells from replicating transferred DNA from engulfed apoptotic bodies [54, 55]. This may be one protection level against the propagation of potentially pathological DNA [54]. It was shown that the ability of cells as recipient of horizontally transferred DNA was enhanced by deficiency of p53 or p21 [54]. It has been suggested that horizontal transfer of DNA from apoptotic bodies could be one explanation to the chromosomal instability observed in cancer cells [54].

CONCLUSIONS

We have attempted to consider the importance of neutrophil efferocytosis within the context of the appropriate immune response to invading pathogens and the inappropriate or excessive inflammation in inflammatory disease. We have mentioned that human gastric adenocarcinoma cells may phagocytose apoptotic neutrophils: a phenomenon similar to that described in efferocytosis of neutrophils during inflammatory reaction but distinct by other types of cell-in-cell phenomena including emperipolesis and entosis both cytologically and biologically. We have highlighted that tumors may take advantage of neutrophil phagocytosis for survival and immune evasion. Where horizontal DNA transfer from apoptotic cells to neoplastic cells, phagocytosing the apoptotic bodies, determine their chromosomal instability, will require further exploration.

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