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Macrophage conditioned medium induced cellular network formation in MCF-7 cells through enhanced tunneling nanotube formation and tunneling nanotube mediated release of viable cytoplasmic fragments

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ABSTRACT

Infiltrating macrophages in tumor microenvironment, through their secreted cytokines and growth factors, regulate several processes of cancer progression such as cancer cell survival, proliferation, invasion, metastasis and angiogenesis. Recently, intercellular cytoplasmic bridges between cancer cells referred as tunneling nanotubes (TNTs) have been recognized as novel mode of intercellular communication between cancer cells. In this study, we investigated the effect of inflammatory mediators present in conditioned medium derived from macrophages on the formation of TNTs in breast adenocarcinoma cells MCF-7. Results show that treatment with macrophage conditioned medium (MϕCM) not only enhanced TNT formation between cells but also stimulated the release of independently migrating viable cytoplasmic fragments, referred to as microplasts, from MCF-7 cells. Time lapse microscopy revealed that microplasts were released from parent cancer cells in extracellular space through formation of TNT-like structures. Mitochondria, vesicles and cytoplasm could be transferred from parent cell body to microplasts through connecting TNTs. The microplasts could also be resorbed into the parent cell body by retraction of the connecting TNTs. Microplast formation inhibited in presence cell migration inhibitor, cytochalasin-B. Notably by utilizing migratory machinery within microplasts, distantly located MCF-7 cells formed several TNT based intercellular connections, leading to formation of physically connected network of cells. Together, these results demonstrate novel role of TNTs in microplast formation, novel modes of TNT formation mediated by microplasts and stimulatory effect of MϕCM on cellular network formation in MCF-7 cells mediated through enhanced TNT and microplast formation.

1. Introduction

Intercellular communication plays a pivotal role in cancer cell proliferation, invasion and metastasis. Cancer cells within tumors are traditionally known to communicate with each other or with stromal cells through secretion of signaling molecules e.g. cytokines, chemokines, growth factors, hormones etc. or by direct cell to cell contact through gap junctions or cellular synapses. Another modality of intercellular communication between cancer cells includes communication through secretion of membrane bound vesicles such as exosomes, mirovesicles or microparticles [1–3]. During the last decade, intercellular communication through formation of intercellular bridges widely referred to as tunneling nanotubes (TNTs) [4–8] or shedding of independently migrating viable cell fragments referred to as microplasts [9] or cytoplasts [10] in cancer progression has gained attention. Based on the potential role of TNTs and viable cytoplasmic fragments in cancer progression [4–10], these communication pathways have emerged as potential therapeutic targets.

TNTs were first reported by Rustom et al. in PC12 cells in 2004 [11]. TNTs are actin based membrane bound cytoplasmic bridges formed between donor and recipient cell, through which wide variety of cellular cargos and signals can be directly transported between distantly connected cells [11]. Several studies have shown the role of TNTs in cancer pathogenesis [4–8,12,13]. For instance, TNTs are shown to spread drug resistant phenotypes in cancer population by mediating intercellular transfer of ABC transporter P-gp [5], mitochondria [6] or miRNA [13]. It has also been reported that horizontal transfer of miRNAs through TNTs from cancer cells to endothelial cells transform endothelial cells towards pathogenic phenotypes which in turn promote cancer cell metastasis [7]. Recently Osswald et al. have

Abbreviations: TNT, Tunneling nanotube; MϕCM, Macrophage conditioned medium; MCM, Monocyte conditioned medium; GM, Growth medium

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also suggested the role of TNT-like structures, which they termed as tumor-microtubes, in spreading radio-resistance in astrocytoma cells in vivo [8]. Migratory cancer cells are shown to form TNTs at the migratory front in scratch wound assays in vitro suggesting their role in cell migration and invasion [4].

Viable cytoplasmic fragments, in contrast, are membrane enclosed viable anucleate large extracellular bodies and were first described in human peripheral leucocytes by Keller and Bessis in 1975 [14]. Later in 1980s, several studies demonstrated the formation of these anucleate viable cytoplasmic fragments in neutrophils cultures and these viable cytoplasmic fragments received various names, such as microplasts [15], cytoplasts [16] and cytokineplasts [17], based on their motility and structural characteristics. These viable cytoplasmic fragments can be differentiated from other membrane bound vesicles such as exosomes and microparticles based on their size, mode of formation and ability of independent migration. Exosomes are 40–150 nm membrane bound vesicles of endocytic origin and microparticles are 50–2000 nm vesicles released by direct outward budding from plasma membrane [3]. Further, both exosomes and microparticles are secreted in body fluids and extracellular fluid as diffusible vectors and move by fluid flow [3]. Viable cytoplasmic fragments, on the other hand, range from 0.5 to tens of microns in diameter, remain adhered to the substrate during formation and after shedding migrate on substrate surface independent of the parent cell [14,15]. While much of the literature available till date has focused on cytoplasmic fragment formation in neutrophils and keratinocytes [14–18], studies on formation and role of viable cytoplasmic fragments and similar structures in cancer cells are limited. Garret et al., reported spontaneous shedding of viable cytoplasmic fragments which they referred as independently migrating microplasts, from human glioblastoma cell lines [9]. The authors also demonstrated that the shedding of independently migrating microplasts from glioblastoma cells was positively correlated with the invasion potential of these cells [9]. Later, shedding of independently migrating cell fragments from human HT-1080 fibrosarcoma cells was also reported [19]. Recently, release of cytoplasmic fragments in vivo from circulating tumor cells have been reported by Headley et al. [10]. The authors have reported that the circulating tumor cells while entering in lung vasculature release independently migrating large microparticles which they termed 'cytoplasts' and that the interaction of these cytoplasts with myeloid cells play a significant role in the metastatic cell seeding.

The studies described here were developed while investigating the effect of macrophage conditioned medium on invasive potential of breast cancer cells and modulation of intercellular communication between cancer cells through TNTs. Many studies suggest that macrophages are most prominent immune cells within tumors [20] and higher macrophage infiltration rates are often associated with poor prognosis and reduced survival [21–23]. Cross-talk between macrophages and tumor cells is mediated through pro-inflammatory cytokines and growth factors secreted by tumor infiltrating macrophages [20]. However, to best of our knowledge, the potential role of macrophages in formation of TNTs in cancer cells has not been explored earlier. Culturing MCF-7 cancer cells in macrophage-derived conditioned medium allows studying the paracrine effect of pro-inflammatory cytokines and growth factors secreted by macrophages on cancer cells in vitro. This culture condition partially mimics the inflammatory tumor microenvironment [24,25] and is known to induce EMT-like phenotype in MCF-7 cells [25]. We observed that treatment with macrophage conditioned medium (MϕCM) not only resulted in enhanced formation of TNTs between MCF-7 cells but also stimulated these cells to shed large number of viable cytoplasmic fragments. Performing time lapse microscopy in macrophage conditioned medium treated MCF-7 cells, we found that these cytoplasmic fragments displayed several morphological characteristics that are typical of microplasts or cytoplasts. Using confocal and time lapse microscopy we have observed that these cytoplasmic fragments are shed from parent cells through formation of TNT-like tubular stalks. We also observed that MCF-7 cells utilized their cytoplasmic fragments connected through the TNT-like tubular stalks to reach out to distantly located cells and mediate formation of intercellular TNTs thereby establishing extensive cellular networks.

2. Materials and methods

2.1. Cell culture

The human breast adenocarcinoma cell line MCF-7 and monocyte cell line U937 were obtained from National Centre for Cell Sciences, Pune, India. MCF-7 was maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic solution (Growth Medium, GM) in humidified atmosphere at 37 °C in 5% CO_2. U937 was maintained in suspension in RPMI-1640 medium supplemented with 10% heat inactivated fetal bovine serum and 1% antibiotic-antimycotic solution (GM) in a humidified atmosphere at 37 °C in 5% CO_2.

2.2. Preparation of conditioned medium

For preparation of monocyte conditioned medium (MCM), ~1×10^6 U937 cells were grown in 4 ml of GM for 24 h. After 24 h the culture supernatant was collected and centrifuged at 5000 rpm for 5 min. The supernatant was filtered using 0.2 μm membrane filters and stored at −20 °C. For preparation of MϕCM, ~1×10^6 U937 cells were treated with 50 nM PMA for 72 h. The adherent cells were washed three times with serum-free medium to remove PMA. Then the adherent cells were further cultured in GM for 24 h. After 24 h, the culture supernatant was collected and centrifuged at 5000 rpm for 5 min. The supernatant was filtered using 0.2 μm membrane filters and stored at −20 °C.

2.3. Quantification of cytokines

The cytokines secreted in the MCM and MϕCM were estimated using inflammatory cytokine ELISArray kit (Qiagen) for human inflammatory cytokines IL1-α, IL1-β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17A, IFN-γ, TNF-α and GMCSF as per the manufacturer's protocol.

2.4. Quantification of TNTs

MCF-7 cells were seeded at a density ~10,000 cells/500 μl/well in 4 well-Labtek-II chambered coverglass in GM and incubated at 37 °C in 5% CO_2 for 24 h. After 24 h the cells were cultured in 50% of MϕCM for 24 h. Control cells were cultured in GM and 50% of MCM. Number of cells and TNTs formed between cells were counted in ~30 random fields in each culture condition using 100X objective lens (UplanFl, 1.3 NA, oil immersion) on inverted microscope (IX70, Olympus, Japan). Each experiment was carried out in triplicates and repeated thrice. At least 300 cells were counted for each culture condition. Results were averaged for number of TNTs per 100 cells to exclude the errors due to difference in cell counts in different culture conditions.

2.5. Quantification of cytoplasmic fragments

MCF-7 cells were seeded at a density of 20,000–40,000 cells in 35 mm tissue culture Petri dishes in GM and incubated at 37 °C in 5% CO_2. After 24 h the cells were cultured in 50% MϕCM for 24 h. Control cells were cultured in GM and 50% MCM. Alternatively, cells were treated with 350 nM cytochalasin-B along with 50% MϕCM or MCM. After 24 h, bright field images of 10 random fields for each culture condition were acquired using 20X objective lens (LCAch, PhC). The number of cell fragments those were still attached to parent cell through TNTs and cell fragments those released from cells were