

Nanotube Formation: A Rapid Form of “Alarm Signaling”?

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ABSTRACT

Purpose: Tunneling nanotubes (TNTs) are extremely thin (50–200 nm), actin-containing cell surface protrusions up to a few microns in length that can develop rapidly and connect various cell types. Mast cells (MCs) are unique immunomodulatory cells that are found perivascularly in all tissues. MCs communicate with many other cell types through the release of inflammatory, neurosensitizing, and vasoactive molecules, through which they are involved in the pathogenesis of many inflammatory diseases. We, therefore, investigated the possibility that MCs may form TNTs and communicate among themselves and with glioblastoma cells.

Methods: Laboratory Allergic Diseases (LAD)-2 human MCs were cultured in medium supplemented with 100 U/mL penicillin/streptomycin and 100 ng/mL recombinant human stem cell factor. They were incubated with 20 nmol/L deep red probe for 20 minutes and 50 nmol/L green probe for 30 minutes. Human glioblastoma cells were incubated with 20 nmol/L deep red probe only, moved to glass-bottom culture dishes, and observed using a substance P 2 confocal microscope. LAD2 MCs were stimulated with 2 μ mol/L of the peptide substance P for 30 minutes at 37°C. Confocal digital images were processed.

Findings: MCs can rapidly (within 5 minutes) form TNTs, which appear to transport mitochondrial and secretory granule particles among themselves and with cocultured glioblastoma cells.

Implications: MCs are now accepted as having an important role in many diseases with an inflammatory component. TNTs provide a rapid and direct way for MCs to “alarm” other cell types with specificity not

present when mediators are secreted into the tissue microenvironment. The identification of TNTs and their cargo could be important in the diagnosis and possible treatment of many inflammatory diseases. (*Clin Ther.* 2016;■:■■■–■■■) © 2016 Elsevier HS Journals, Inc. All rights reserved.

Key words: inflammation, mast cells, pathogenesis, tunneling nanotubes.

INTRODUCTION

All types of cells communicate in different ways that include the secretion of chemokines, cytokines, and hormones, as well as calcium and neurotransmitters.¹ Neurons, glial cells, T cells, and even mast cells (MCs) can also communicate through gap junctions and synapses.^{2,3} However, these processes have little selectivity, require considerable time for secretion, or are constrained by intricate cell-to-cell contact. An impressive number of publications report the ability of cells to communicate directly through the formation of tunneling nanotubes (TNTs), tiny (50–200 nm in width), F actin-based, membranous projections that can transport numerous diverse molecules, including nucleic acids and proteins.^{4–8} The presence of TNTs could have implications for the diagnosis and treatment of many diseases.⁹

MCs derive from bone marrow progenitors that develop different tissue-specific phenotypes, depending

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on the microenvironment, and are involved in allergic reactions,¹⁰ mastocytosis,¹⁰ and MC activation syndrome.^{11,12} Once activated, MCs secrete numerous vasoactive, neurosensitizing, and proinflammatory mediators, including histamine, prostaglandins, and tryptase, as well as cytokines (interleukins 1, 4, 6, 9, 13, and 33, and tumor necrosis factor) and chemokines (chemokine [C-X-C motif] ligand 8, and chemokine ligands 2 and 5).¹⁰ Unfortunately, most of these molecules are secreted into the microenvironment without any specificity and are subjected to rapid degradation.

We have shown that MC activation leads to mitochondrial translocation to the cell surface¹³ and to extracellular secretion of mitochondrial DNA,¹⁴ which augments MC activation.¹⁵ Extracellular mitochondrial DNA¹⁶ could be misidentified by the body as an innate pathogen, leading to autoinflammatory responses¹⁴ that, along with oxidative stress, could contribute to many inflammatory diseases and to aging.¹⁷ One way to avoid such autoinflammatory responses would be to deliver mitochondrial DNA and other relevant molecules within TNTs. For instance, it was recently shown that healthy PC12 neuroblastoma cells could transfer intact functional mitochondria to UV-damaged PC12 cells and salvage them.¹⁸

MCs are now accepted as having an important role in homeostasis,¹⁹ immunity,²⁰ infection,²¹ and inflammatory processes²² that are a causal factor in many diseases,²³ including autism spectrum disorder (ASD),²⁴ cancer,²⁵ coronary artery disease,²⁶ fibromyalgia,²⁷ interstitial cystitis,²⁸ and psoriasis.²⁹ Here we report the rapid formation of TNTs by cultured MCs and glioblastoma cells from humans. We include enough details of the methodology for others to replicate our findings.

MATERIALS AND METHODS

Culture of LAD2 MCs

LAD2 cells (supplied by Dr. A.S. Kirshenbaum, National Institutes of Health, Bethesda, MD), derived from a human MC leukemia,¹⁴ were cultured in StemPro-34 medium (InvitroGen, Carlsbad, California) supplemented with 100 U/mL penicillin/streptomycin and 100 ng/mL recombinant human stem cell factor (supplied by Biovitrum AB, Stockholm, Sweden).

Confocal Microscopy

LAD2 cells were incubated with 20 nmol/L MitoTracker Deep Red probe (InvitroGen) for 20 minutes

and 50 nmol/L LysoTracker DND Green probe (InvitroGen) for 30 minutes. Glioblastoma cells were incubated with 20 nmol/L MitoTracker Deep Red probe (InvitroGen) only, moved to glass-bottom culture dishes (MatTek, Ashland, Massachusetts), and observed using a Leica TCS SP2 confocal microscope (Leica Microsystems, Buffalo Grove, Illinois). LAD2 cells were stimulated with substance P (Sigma-Aldrich, St. Louis, Missouri; 2 μ mol/L for 30 minutes at 37°C). Confocal digital images were processed using ImageJ software version 1.32 mitochondria calculator plug-in (National Institutes of Health, Bethesda, Maryland)²⁹ and Photoshop software version 7.0 (Adobe Systems, San Jose, California).

RESULTS

In one set of experiments, human LAD2 MCs were incubated with MitoTracker Deep Red (InvitroGen; 20 nmol/L, 15 minutes) to distinguish mitochondria as described previously.¹³ Cells were washed and allowed to settle in glass-bottom dishes (P35G-0.170-14-C; MatTek). MCs were then treated with the F actin-depolymerizing agent latrunculin B (Invitrogen; 10 μ mol/L) and observed immediately with a Leica TCS SP2 confocal microscope (Leica Microsystems) under either reflected light mode (**Figure 1A**) or confocal mode (**Figures 1B and 1C**). Latrunculin B was associated with rapid (1–5 minutes) formation of numerous membranous projections (**Figure 1A**) that would qualify as TNTs by virtue of the fact that they contained punctate red color, indicating the presence of mitochondrial particles (**Figures 1A–1C**).

In another set of experiments, MCs were incubated with LysoTracker DND (Invitrogen; 100 nmol/L, 30 minutes), which stains the secretory granules green, and MitoTracker Deep Red (20 nmol/L, 15 minutes). Cells were washed and allowed to settle in glass-bottom dishes (MatTek) before observation under confocal mode as described earlier. Treatment of MCs with latrunculin B (10 μ mol/L) was associated with rapid (1–5 minutes) formation of numerous TNTs containing both punctate green (secretory granules) and red color (mitochondrial particles), which appeared to have made connections with other MCs (**Figures 1D–1F**). Stimulation of MCs with substance P (known to induce MC mediator release) did not have any apparent effect on the formation of TNTs (results not shown).

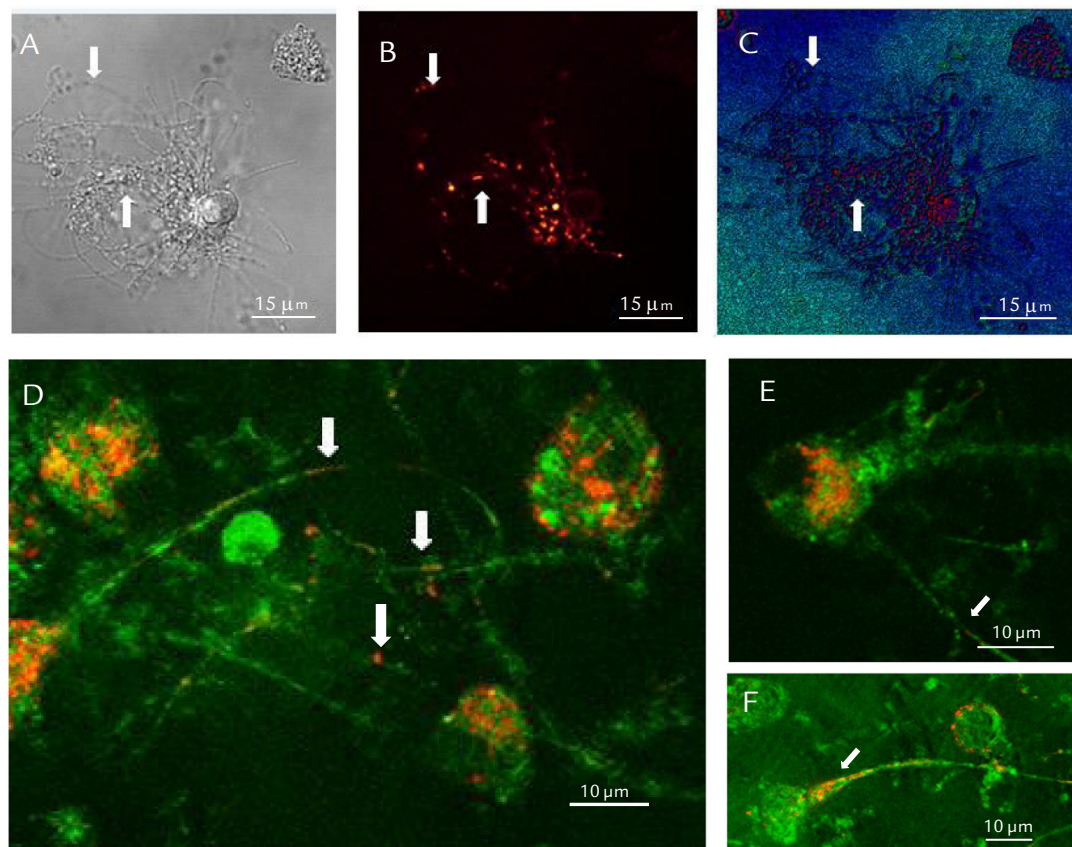


Figure 1. Photomicrographs of mast cells (MCs) with tunneling nanotubes (TNTs). Laboratory Allergic Diseases (LAD)-2 MCs were incubated with MitoTracker Deep Red (InvitroGen, Carlsbad, California; 20 nmol/L, 15 minutes) before treatment with latrunculin B (10 μ mol/L, 15 minutes). MCs were observed with Leica TCS substance P (SP)-2 Confocal microscope (Leica Microsystems, Buffalo Grove, Illinois) under either reflected light mode (A) or confocal mode (B and C). Numerous membranous projections are evident (A, red), indicating the presence of mitochondrial particles (B and C, arrows). D–F, MCs were also incubated with LysoTracker DND (InvitroGen; 100 nmol/L, 30 minutes) to distinguish secretory granules before treatment with latrunculin B (10 μ mol/L, up to 15 minutes).

MC–microglia interactions have been reported to be involved in brain inflammation.³⁰ We, therefore, cocultured MCs with U251 human glioblastoma cells which are known to release exosomes containing mitochondrial DNA.³¹ U251 human glioblastoma cells were grown as monolayers in glass-bottom dishes (MatTek) until 50% confluent before staining with MitoTracker Deep Red as described previously. MCs were stained with LysoTracker DND as described earlier and then carefully layered onto the U251 glioblastoma cell monolayer in the dish and allowed

to settle before observation under confocal microscopy. MCs cocultured with glioblastoma cells did not generate TNTs (Figures 2A and 2B). However, when the cocultured cells were treated with latrunculin B (10 μ mol/L), there was rapid formation of TNTs containing punctate mitochondrial particles, apparently originating from the glioblastoma cells (Figure 2C). Some of these TNTs also contained secretory granule particles (Figure 2D), indicating bidirectional communication between the 2 different cell types.

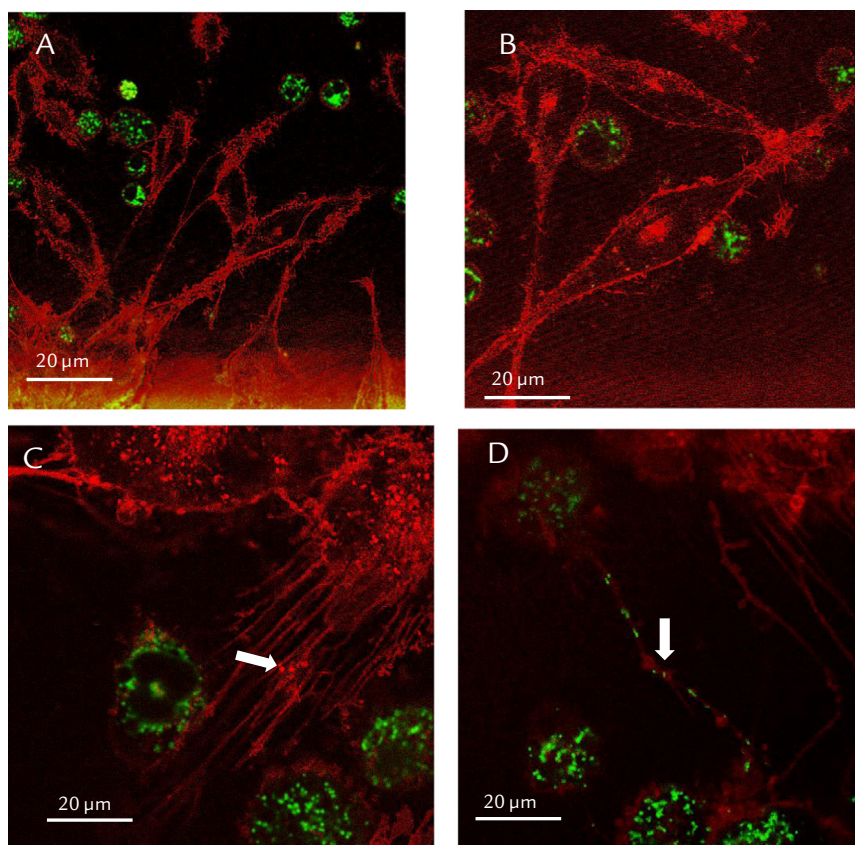


Figure 2. Photomicrographs of mast cells (MCs) with tunneling nanotubes (TNTs) making contact with glioblastoma cells. U251 glioblastoma cells grown in monolayers were incubated with MitoTracker Deep Red (InvitroGen, Carlsbad, California; 20 nmol/L, 15 minutes), and MCs were stained with LysoTracker DND (InvitroGen; 100 nmol/L, 30 minutes); MCs were then gently layered on top of the glioblastoma cells (A and B). The cocultured cells were then treated with latrunculin B (10 μ mol/L, 15 minutes). Multiple MitoTracker-positive (red) TNTs containing punctate mitochondrial particles (C, arrow) are evident. Some of these TNTs also contain secretory granule (green) particles (D, arrow), connecting the two different cell types.

DISCUSSION

The present findings show that MCs and glioblastoma cells in humans can rapidly form TNTs that contain mitochondrial and secretory granule material. Such TNTs may constitute a stress response, communicating information to neighboring cells such, as shown in healthy PC12 neuroblastoma cells that transferred functional mitochondria to UV-damaged PC12 cells and rescued them.¹⁸ Moreover, endothelial cells could carry mitochondria to cancer cells modulating chemoresistance.³²

Latrunculin-like HIV virus negative factor (Nef) protein induced the formation of TNTs, through

which they were injected into cells, and controlled their growth and survival.³³ Nanotube connections between T cells apparently permit HIV-1 transmission.³⁴ On the other hand, nanotubes transferred the antiapoptotic protein MCL-1 myeloid cell leukemia-1 from airway smooth muscle cells to CD⁺ T cells and promoted their survival.³⁵ Malignant mesothelioma cells have been shown to form nanotubule connections in situ.³⁶

TNT formation could be important during abnormal microglia activation and proliferation, which could lead to focal inflammation and “choking” of normal synaptic traffic.³⁷ Microglia dysfunction and

neuroinflammation have been reported in the brains of patients with ASD.^{38–40} A recent study of the transcriptomes from 104 samples of brain cortical tissue from patients with ASD identified gene clusters associated with increased microglia activation and decreased neuronal activity.⁴¹ As a result, microglia are now considered an important component of the pathogenesis of ASD.^{42,43} MCs are important in neuroinflammation.^{44,45} Stimulation of brain MCs in mice was recently shown to induce microglia activation and brain inflammation, inhibited by an MC stabilizer.⁴⁶ MC-derived histamine⁴⁷ and tryptase⁴⁸ can stimulate microglia, a finding that has led to the proposal that MC–microglia interactions are important in neuroinflammation.^{46,49} Our present findings of TNTs between MCs and glioblastoma cells make it possible that TNTs may form between MCs and microglia.

We first discovered that MCs can secrete the content of individual granules,⁵⁰ as well as individual molecules, selectively without degranulation and without secretion of either histamine and tryptase.⁵¹ We specifically showed that interleukin 1 can stimulate selective release of interleukin 6⁵² and that corticotropin-releasing hormone can selectively release vascular endothelial growth factor.⁵³ It has since been shown that MCs can secrete a number of mediators selectively without degranulation.⁵⁴ MCs can also communicate with neurons by transgranulation.⁵⁵ Moreover, xxx can undergo polarized exocytosis of proteolytic enzymes in what has been termed an *antibody-dependent degranulation synapse*.⁵⁶ MCs can also secrete phospholipid nanovesicles (exosomes)⁵⁷ that could carry a number of biologically active molecules⁵⁸ in a manner guided by surface antigens.⁵⁹ Such exosomes may be involved in neuropsychiatric diseases.^{60,61}

TNTs might allow MCs to be involved in the diverse pathophysiological processes of many seemingly diverse diseases.²³ TNTs may even regulate MC activation and coordinate their recently discovered circadian clock reactivity.^{62–64} The identification of TNTs and the molecules they transport could be of diagnostic and therapeutic importance in especially inflammatory diseases.^{9,65,66}

CONCLUSIONS

MCs are now accepted as having an important role in many diseases with an inflammatory component.

TNTs provide a rapid and direct way for MCs to “alarm” other cell types with specificity not present when mediators are secreted into the tissue micro-environment. The identification of TNTs and their cargo could be important in the diagnosis and possible treatment of many inflammatory diseases.

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Dr. Theoharides conceived and wrote the manuscript. Z. Weng and B. Zhang performed the experiments. I. Tsilioni searched the literature and contributed with the writing.

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CONFLICTS OF INTEREST

Dr. Theoharides has been awarded US patent number 8,268,365, “Anti-Inflammatory Compositions for Treating Brain Inflammation.” The authors have indicated that they have no other conflicts of interest with regard to the content of this article.

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