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The Effects of Epigallocatechin Gallate on the Structure and Dynamics of Podosomes in Human Macrophages

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THE EFFECTS OF EPIGALLOCATECHIN GALLATE ON THE STRUCTURE AND DYNAMICS OF PODOSOMES IN HUMAN MACROPHAGES

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Abstract:

Podosomes are actin rich structures found in cancer cells and cells derived from monocytes in the human body. They help these cells be motile through the body and invade through tissues. We found the number of podosomes observed in the cells increased, with a simultaneous increase in motility, corresponding to an increase in the concentration of Epigallocatechin gallate(EGCG) used in a dose-dependent fashion. We also found that the actin molecules in the podosomes are arranged in columns with frequent breaks in its structure. We also found myosin IIb to be an integral part of the podosome, providing the podosome with the banding pattern along with actin. We were also able to confirm the presence of gelsolin in the podosome and assembled together a model trying to explain the interaction among all these proteins.

Introduction:

With cancer soon becoming the leading cause of death across the world, it has become important to understand the mechanisms that guide it, in order to be able to stop its spread in the body. The cancer spreads throughout the body by the process of metastasis, and during this process, cells which are usually dormant get transformed and become motile and invasive. These cancers cells accomplish the task of moving through tissues by employing the aide of specialized structures in the cells called podosomes.

Podosomes are actin rich structures and are found only in cells derived from monocytes (example: macrophages, osteoclasts etc.) and, as mentioned, in cancerous cells, making them unique structures in cellular biology (Buccione, Orth & McNiven, 2004; Linder & Aepfelbacher, 2003; Petit & Thiery, 2000). Podosomes are highly dynamic structures in cells and have an average half life of two to twenty seconds (Evans & Matsudaria, 2006). Though the overall structure of the podosomes might be the same in the variety of cell lines in which they are found, they show a difference in orientation and organization in these cell lines (Gimona & Buccione, 2006). There is minimal knowledge about their structural composition and hence, to study these podosomes we used human macrophages. Macrophages are cells that get rid of foreign and/or diseased elements in the body by actively consuming them. They, like cancerous cells, are highly motile and utilize podosomes to move into and invade new tissues to

reach sites of action (Yamaguchi, Pixley & Condeelis, 2006; Zhang, Southwick & Purich, 2002). Podosomes and invadopodia (podosome-like structures present in these cells) use the same actin structures and have very similar signaling molecules in their development. The difference between the two structures is the presence or absence of an invasive sub-structure (Gimona, Grashoff & Kopp, 2005). Recent studies show that the difference between podosomes and invadodia is dependent upon matrix stiffness, i.e. podosomes would progress to becoming invadopodia if they could invade the tissue (Discher, Janmey & Wang, 2005). Therefore, in an experimental system that cultures cells on a solid substrate, podosomes may not differentiate into their invasive form, i.e. invadopodia.

Recent studies have also shown a link between cancer prevention and green tea (Dufresne and Farnworth, 2001). In previous experiments we found that the desirable effects of green tea could be mimicked by just using EGCG, the active polyphenol present in green tea (Graham, 1992). Presently, EGCG is being researched as a chemo-preventative agent for cancers of different types and in different cell types (Beltz, Byer, Moss & Simet, 2006). EGCG also affects the activity of the Rho family of proteins; these act as intra-cellular switches (Lu et al., 2005; Hall, 1998). Members of the Rho family, especially, Rho, Rac and Cdc42 have been shown to be central constituents in the metabolic pathway leading to actin assembly (Hall & Nobes, 2000). Hence, any change in the

activity of these proteins by EGCG would lead to changes in the actin structures i.e. the podosomes.

In past experiments we found an association between the motility of macrophages and the concentration of EGCG used. In this series of experiments we tried to decipher the role of podosomes in the motility of the macrophages. At the same time, we also worked on trying to determine the

structural components of the podosomes. Different models have been proposed for the structure of podosomes. One prominent model was proposed by Stephan Linder and his colleagues (Linder & Kopp, 2005). Building upon his model, we would like to confirm or deny the presence of the relevant proteins in our experimental macrophages. These include: the Arp2/3 complex, Gelsolin, Myosin II and Rho GTPases (Figure 1). We also plan to look at their involvement in the structure of the podosomes to understand how these proteins are affected by the presence of EGCG and the pathways that lead to their development.

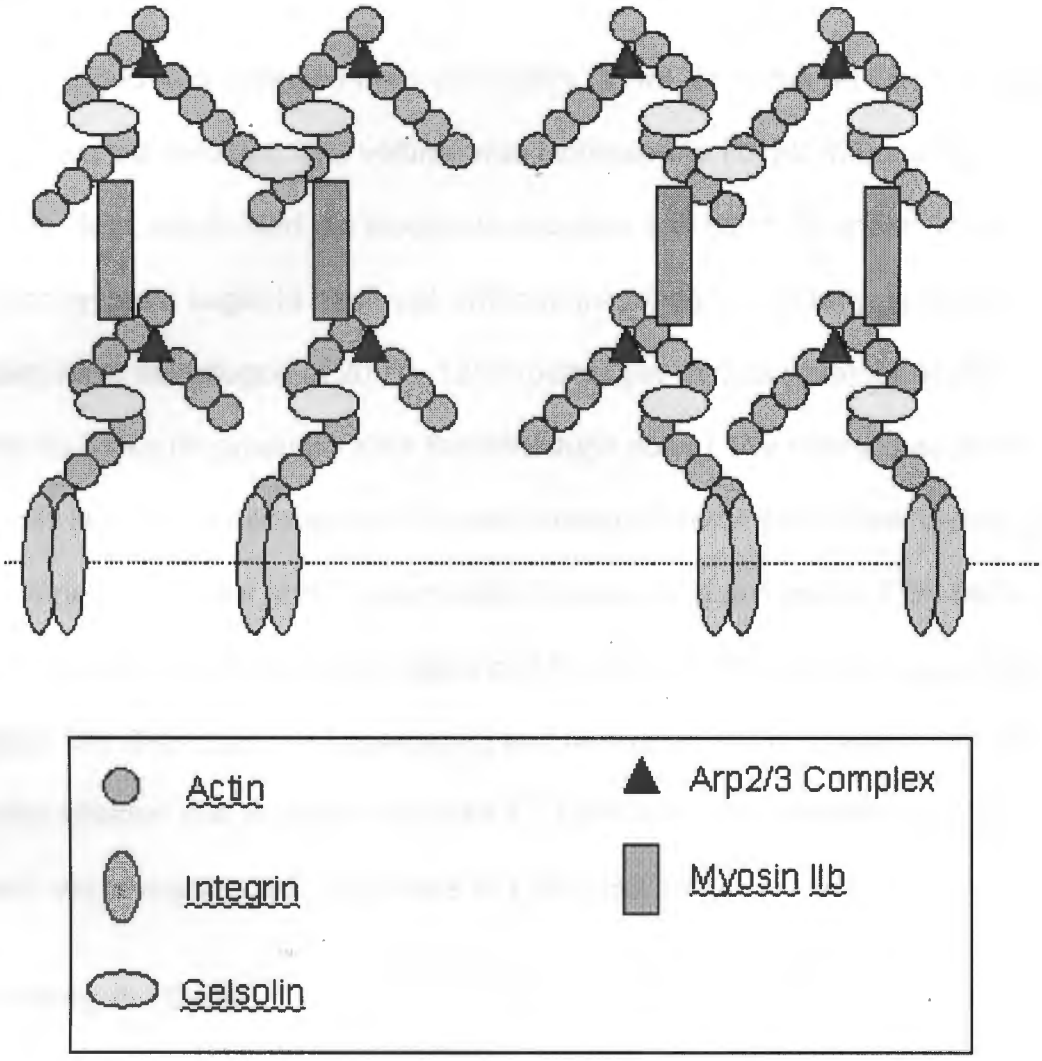

Figure 1. *Proposed Model of the Podosome.*

Methods:

Gathering Blood:

Blood was collected from volunteers, 30 mL at a time with 5 mL heparin, by a certified collector. The volume was increased to 70 mL with sterile PBS buffer. Next, we divided the blood into two vials and carefully added 15 mL Phycohyyphae sugar to each vial, without disturbing the underlying layers. The tubes were centrifuged at 20 °C, 1300 rounds per minute (rpm) for 45 minutes with no brake (to gradually slow the centrifuge down). We then extracted the cloudy layer of monocytes form the vial without disturbing the other layers. We combined the layers from the two vials (if necessary) and added PBS buffer to fill the tube and centrifuged them again at 4 °C, 1200 rpm for 7 minutes with full brake. We discarded the supernatant and re-suspended the pellet in the PBS buffer solution and re-centrifuged at 4°C, 1200 rpm for 7 minutes with full brake. Next, we re-suspended the pellet/s in 1 mL PBS buffer.

Growing the Cells:

The concentration of the monocytes in the solution was established using a Hemocytometer. The cells were visualized using Eosin stain. To proceed, we used a minimum concentration of $2X10^6$ cells/ mL on average. We transferred the cells onto an eight-chamber slide with 500uL in each chamber. We then incubated the slide at 37 °C for one hour.

Wound Healing/ Scratch Test:

We transferred the cells onto 1.5 cm Petri dishes and incubated them for 45 minutes at 37 °C. The dishes were then scratched a couple times each with a sterile pipette tip and exposed to the specific concentration of EGCG and incubated again for 18 hours at 37 °C. We then counted the number of cells that had migrated into the scratches.

EGCG Exposure:

We discarded the supernatant carefully, without disturbing the bottom cellular layer and proceeded to carefully wash the cells with sterile PBS buffer twice. We then added the appropriate concentration of EGCG to the chambers and let the cells incubate overnight at 45 °C.

Preparation for Staining:

Before beginning we made sure that all the solutions are at room temperature. We washed the cells, with gentle agitation, with PBS buffer only once over ice without disturbing the cells. We fixed the cells with 3.7 % formaldehyde onto the slide using the modified protocol by Julie Ceils (pg. 352, 1994), at room temperature, for 10 minutes. We washed the cells with sterile PBS buffer four times for 2 minutes each with gentle agitation. We added the Homemade Blocking Solution (HBS) (80 mL sterile PBS buffer+ 1 ml Tween-20 + 1g non-fat

dairy milk. Bring the volume to 100 mL with sterile PBS buffer) for 30 minutes and removed the HBS. The cells were now fixed and permeabalized.

Actin Staining:

(The following steps were done in the hood with proper protection and in the dark.) We added 50uL of Phalloidin solution (5uL of phalloidin stock in 1ml 1% DMSO in PBS) with excitation wavelength of 515 nm, to all the chambers, incubated in dark at 37 °C for 45 minutes. All the steps following Phalloidin staining were done in the dark. The cells were then washed with gentle agitation four times for 3 minutes each.

Second Staining:

We added 50 uL of the Primary Anti-body to the chambers and incubated the slide at 37 °C for 45 minutes. We then washed the cells with 1 % Tween-20 in sterile PBS buffer three times for 2 minutes each. We then added the corresponding Secondary Anti-body to the chambers and incubated the slide at 37 °C for 45 minutes. We then washed the cells with sterile PBS buffer three times for 2 minutes each.

We then carefully removed the chambers and the well gasket off the slide using a razor blade and forceps making sure to not disturb the fixed cells. We added a drop of Molwiol anti-fade mounting solution to each chamber and gently

pressed down the coverslip. We allowed the slide to dry overnight and stored it wrapped in aluminum foil in the fridge at 4 °C to retard fading.

Imaging and 3D Reconstruction:

Once the slide was ready, we viewed the cells using an inverted fluorescent microscope equipped with the appropriate ultra-violet excitation radiation and also the filters corresponding to the wavelengths of the specifically labeled molecules. The microscope and associated camera were controlled via a computer interface that took images in stacks along the vertical (focal) axis, usually 0.15 um apart (1.5 to 2.5 um). These images were then put together using the ImageJ software which produced a 3-D representation of the structures for analysis. These images allowed further analysis regarding the structural columns of actin and the other proteins and their sequential nature as related to experimental treatment.

Results:

Effect of EGCG on the Macrophages:

We began by analyzing the number of macrophages which were able to migrate by doing a Wound Healing/ Scratch Test. The data in Table 1 suggests that fewer macrophages move around with an increase in the concentration of EGCG used.

Table 1. Results of The Wound Healing/ Scratch Test.

EGCG	0uM	10uM	20uM	40uM	80uM
Number of Cells in Scratch	50	48	35	33	30
	63	56	32	29	21
	69	54	35	35	28
Mean Number of Cells	60.3	52.7	34.0	32.3	26.3

We also saw a decreasing number of cells in the slide chambers with increasing concentrations of EGCG. This led us to analyze the concentrations of cells in the solution versus the concentration of the cells fixed on the plate. We saw that the number of cells that were found in solution had increased as shown in Table 2, corresponding to an increase in the concentration of EGCG used.

Therefore, data from these two tables put together tell us that even though the macrophages are becoming less motile along the plate surface, they are being more readily detached.

Table 2. Number of Cells / mL From the Wound Healing/Scratch Test.

	Attached Cells			Loose Cells		
EGCG	0uM	20uM	40Um	0uM	20Um	40Um
Conc. Of Cells / mL	0.548	0.52	0.412	0.756	0.808	1.213
	0.537	0.552	0.435	0.752	0.937	1.305
	0.541	0.572	0.458	0.687	0.956	1.259
Mean Conc. of Cells	0.542	0.553	0.444	0.746	0.920	1.246

We then analyzed the cells phalloidin stained actin sections and found a dose-dependent increase in the number of bright spots the cells that correspond to podosomes as seen in Figure 2. We, therefore, speculate that the increasing dose of EGCG leads to an increase in podosomes that causes the cell to become reluctant to move but at the same time, increased its detachment from the membrane it is present on.

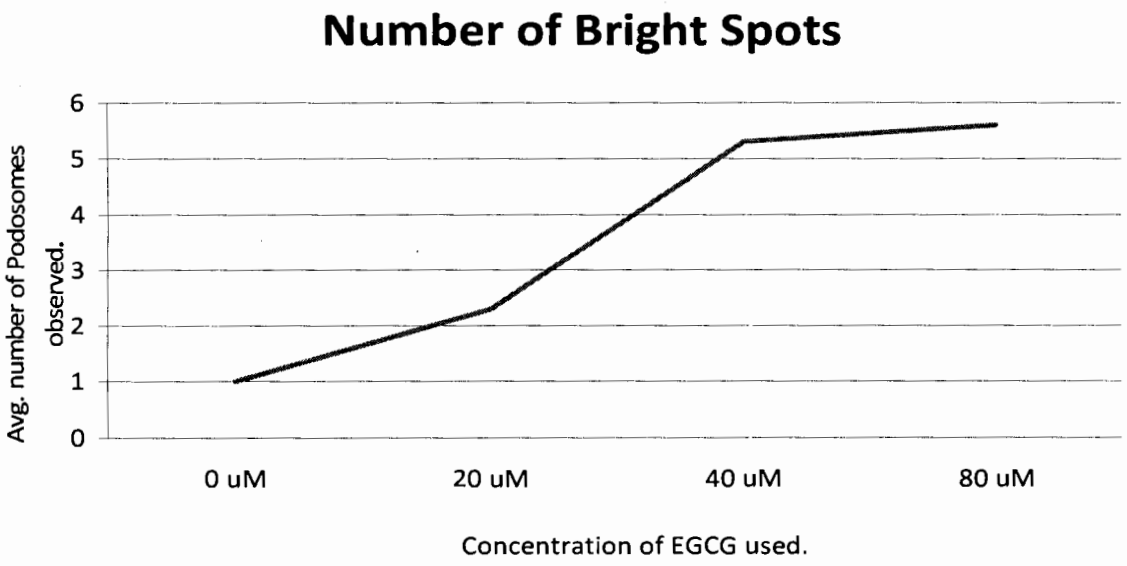

Figure 2. *Graph showing the number of bright spots corresponding to podosomes observed in relation to the concentration of EGCG the cells were exposed to.*

Recent studies have probed into this feature of the podosomes and found that the podosomes are responsible for accumulating Matrix Metalloproteins (MMP) that degrades the extracellular matrix in its vicinity. This role has usually been ascribed to invadopodia, but recent studies show that podosomes and invadopodia are more similar than was previously thought. They are very similar to each other in their structure and composition, with the invadopodia forming a protusion, which helps it break the matrix more easily (Linder, 2003).

Actin Staining: As was mentioned earlier, actin forms an integral part of the cell membrane as well as the podosomes, staining the cells with actin led to many valuable insights into the effects of EGCG on the cells. As the concentration of the EGCG used increased we saw the cells decrease in size but at the same time, we also saw the cell membrane become more regular (Figure 3).

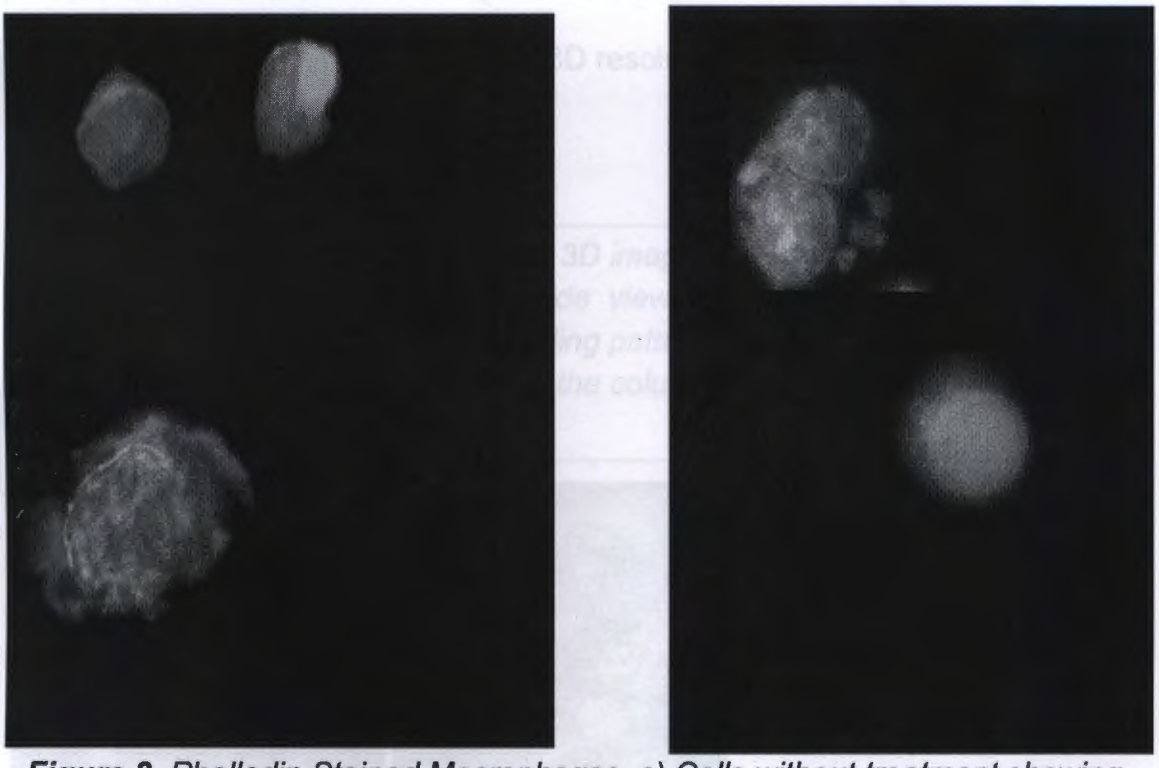

Figure 3. *Phallodin Stained Macrophages. a) Cells without treatment showing more dynamic exoskeleton, with fewer podosomes (bright green spots). b) Cells treated with 40uM EGCG showing a less dynamic cytoskeleton but more podosomes.*

This led us to believe that as the concentration of the EGCG the cells are exposed to is increased, the cells became more prepared to migrate through the membrane. Therefore, the increased podosomes would lead to an increase in matrixmetallo protein (MMP) secretion, which would help the cell travel through the tissue more easily and reach the required site, helped by its small more compact geometry. 3D analysis of the podosomes showed no difference in the podosome itself with an increasing concentration of EGCG. Only a change in the number of podosomes was observed. 3D resolution of the slides also showed

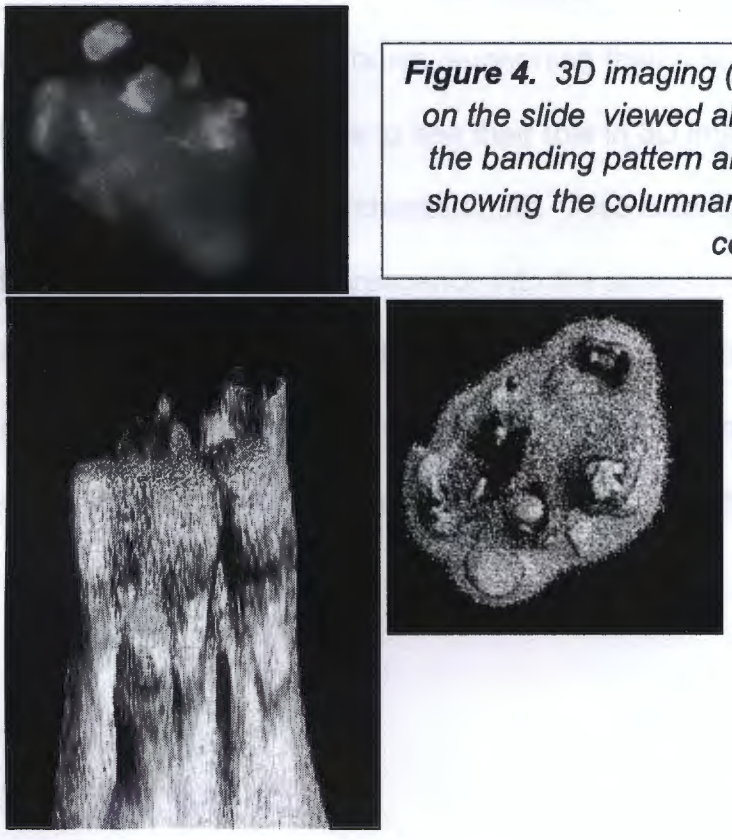

Figure 4. *3D imaging (going counterclockwise) on the slide viewed along the z axis showing the banding pattern and the third, a top-view showing the columnar podosomes inside the cell.*

that podosomes were formed of actin dense structures, with a banding pattern

observed in it (Figure 4). The banding patters were initially thought to be an artifact of the slicing technique, but we found that the banding pattern was similar despite varying the distance between the slices. This proved that the banding pattern was integral to the podosome and led us to speculate the integration of myosin proteins in the structure of the podosome, similar to that in muscle cells.

Gelsolin Staining: We stained the gelsolin in macrophages with Mouse anti-gelsolin and used anti-mouse Rhodamine (excitation wavelength of 552nm) to visualize the gelsolin proteins. As per our previous observations, we saw a co-localization of gelsolin with the actin polymers (Fig. 5). We also found that they were present in large amounts, supporting their role as F-actin capping proteins. However, we were unable to see their role in 3D imaging due to their unpredictably fast fading characteristic. Also complicating the issue was the bleeding of phallodin fluorescence into the analysis (during the process taking 3D slices, the actin stain would show up and complicate the results). Due to the same reason we could not verify if gelsolin was present at two locations in the cells, i.e. along the core and at the boundary of the base as shown in Figure1.

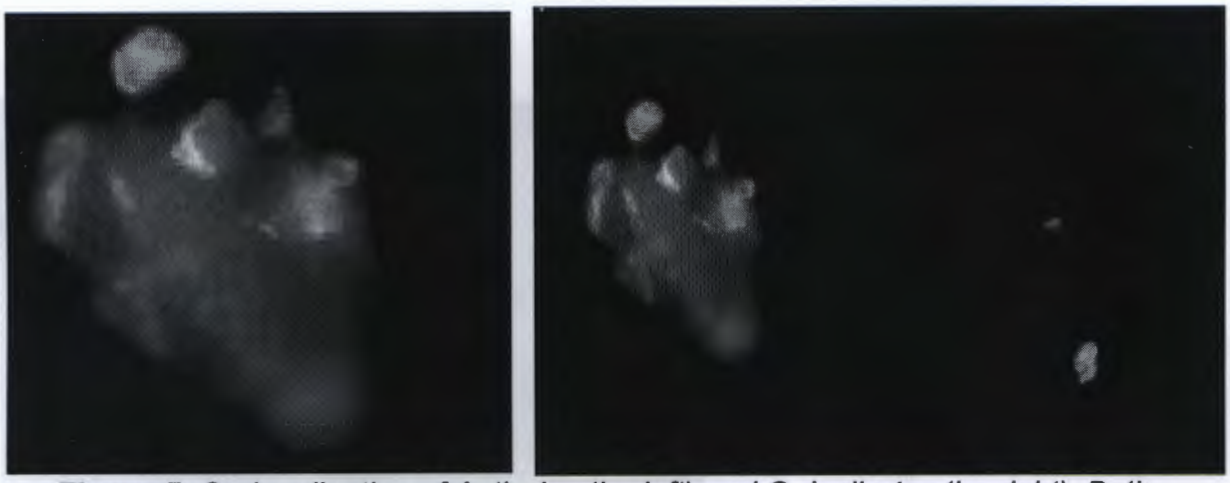

Figure 5. *Co-localization of Actin (on the left) and Gelsolin (on the right). Both are found at the podosomes represented by the bright spots.*

Myosin IIb Staining: We stained myosin with mouse anti-Myosin IIb (obtained from Developmental Studies Hybridoma Bank, University of Iowa) and used Qdot 585 goat F(ab')2 anti-mouse IgG Conjugate (excitation wavelength of 586nm) to visualize the myosin proteins. Early experiments showed that myosin and actin co-localize at the podosome structures (Figure 6) and our results prove this. We obtained very few images of the Qdot label due to its accelerated fading. This also made it difficult for us to visualize the 3D geometry of the myosin proteins' interaction in the podosome. But our preliminary findings indicate that myosin IIb is present in high concentrations in the podosomes and that they are found in the actin columns. They are also localized at the base of the podosome and at higher levels on the podosome, though we did not always see the second. We believe that the myosin IIb proteins give the podosomal structures contractile ability, helping it engage the cytoskeleton to prepare it for the move corresponding to its dynamic nature.

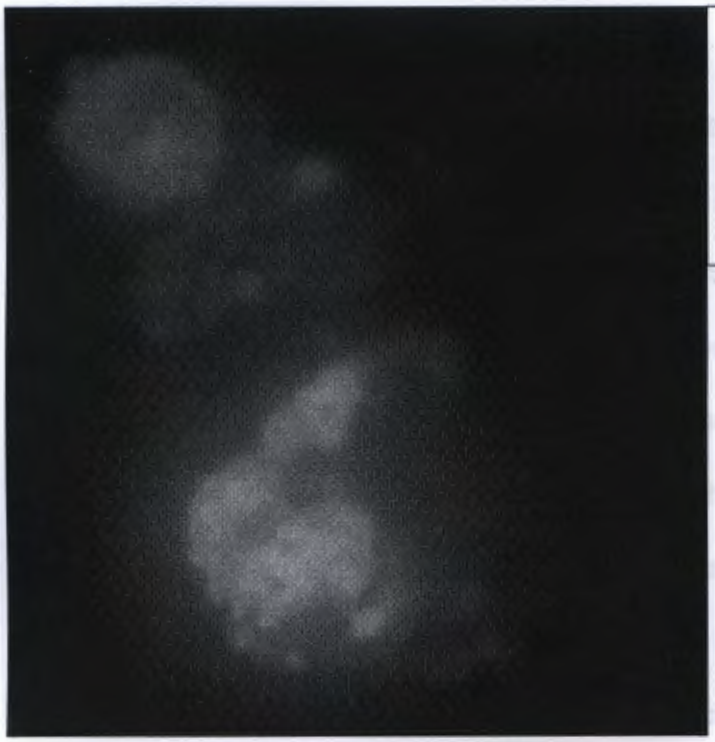

***Figure 6**. Antibody Staining showing the presence of myosin IIb, represented by the bright orange spots in the macrophages.*

Other Proteins: Due to time restraints, we were unable to test the presence and involvement of the other proteins in the podosome that we proposed to study. The fading out of the secondary antibodies in both the tests discouraged us to carry forward newer experiments without resolving the issue first.

Conclusion:

We began by showing an association between the appearance of the podosome in the macrophages and the amount of EGCG used. We found that the cells were more motile with an increase in the concentration EGCG and this also corelated to an increase in the number of podosomes in each cell. We then looked at the structure of the podosomes and found that the actin formed a major fraction of the podosomal columns. They also showed a characteristic banding pattern similar to that in muscle cells. This led us to observing myosin Iib in the podosomes co-localized with the actin. We also found gelsolin to be involved in the podosome. This data combined helps us picture the structure of a podosome as shown in Figure 1, though we still have not been able to confirm the 3D orientation of all the proteins studied. We were also not able to look at the role of RhoGTPases in these cells or establish the point in the cellular pathway that gets affected by EGCG. Further research needs to be done to decipher the pathways involved, inoreder to be able to understand the role of the podosomes and subesequently, have treatments against cancer, directed at the podosomes.

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