Shedding of microparticles by myofibroblasts as mediator of cellular cross-talk during normal wound healing

Véronique J. MOULIN, PhD[1, 2*], Dominique MAYRAND, Msc[1], Hugo MESSIER, MD[1], Maria Carmen MARTINEZ, PhD[3], Carlos A. LOPEZ-VALLÉ, MD[4] and Hervé GENEST, MD[5]

[1] LOEX, Centre de Recherche FRSQ du Centre Hospitalier Affilié Universitaire de Québec, 1401, 18e rue, Quebec, Canada, [2]Department of Surgery, Laval University, Quebec, Canada, [3]CNRS UMR 6214-INSERM 771, Université d'Angers, Angers, France, [4]Complexe Hospitalier de la Sagamie, Chicoutimi, Canada, [5]Centre Hospitalier Affilié Universitaire de Québec, Quebec, Canada

Corresponding author: Véronique Moulin, Ph.D.
LOEX, Hôpital de l'Enfant-Jésus
1401, 18e rue
G1J 1Z4 Québec (QC) Canada
Phone number : (418) 990-8255 #1715
Fax number : (418) 990-8251
Email : veronique.moulin@chg.ulaval.ca

RUNNING HEAD: Wound myofibroblasts produce microparticles

KEYWORDS:

* **Wound healing**
- **myofibroblast**
- **microparticle**
- **hypertrophic scar**
- **FGF**
- **VEGF**
- **serum**

Total number of text figures: 6

Grant information: contract grant sponsor: CIHR, contract grant number: MOP#84280

ABSTRACT

Interactions between cells are a crucial mechanism to correctly heal a wounded tissue. Myofibroblasts have a central role during healing but their means to communicate with other cells is unknown. Microparticles (MP) have demonstrated a potential role as mediators of cellular interactions during various diseases. We have analyzed the production of MP by normal (Wmyo) and pathological (hypertrophic scar, Hmyo) myofibroblasts and human dermal fibroblasts (Fb) when treated with serum or plasma as examples of body fluids. We have shown that the presence of these body fluids induced a very significant increase in MP production by Wmyo while no MP production was denoted for Hmyo and Fb. These effects were at least due to thermally sensitive protein(s) with a molecular mass greater than 30 kDa. Furthermore, the increase in MP production was not linked to an increase in apoptotic Wmyo. MP characterization showed that VEGF and FGF2 were present in MP and that endothelial and (myo)fibroblast cell growth can be stimulated by MP treatment. We postulated that MP production by myofibroblasts could modulate mesenchymal cell growth and angiogenesis during normal healing.

INTRODUCTION

Shedding of microparticles (MP) is a vital phenomenon frequently observed during cell activation and apoptosis (Freyssinet, 2003). These vesicles can be released by virtually all cell types but endothelial, blood, smooth muscle and cancer cells are the most studied cell types concerning the capacity to produce MP (Martinez et al., 2005). Although MP were long considered as cellular debris with no biological significance, recent studies have demonstrated a potential role of these structures as mediators of cellular interactions (Martinez et al., 2005; Millimaggi et al., 2007). MP can thus transfer bioactive molecules from one cell to another and serve as subcellular vectors to propagate signals. They have been shown to have significant roles in vascular dysfunction (Martinez et al., 2005) (Agouni et al., 2008), in inflammation (Distler et al., 2006) or during abnormal fibrosis (Jiang et al., 2009). Furthermore, their increased concentration in blood of patients under pathological states such as immune disorders (Distler et al., 2006) or vascular diseases (Boulanger et al., 2006), strengthens the notion that MP may play a role in numerous diseases. On the other hand, even if MP are present in blood of healthy individuals, few studies have shown a role of MP during normal physiological mechanisms (Freyssinet, 2003).

Wound healing is a complex biological process where numerous cell types play a role. The interactions between each cell populations are crucial to correctly regulate each phase of the healing process (Moulin, 1995). Whereas inflammatory cells have the major role during the inflammation phase, endothelial cells, epithelial cells and myofibroblasts are the main actors of the granulation tissue phase. Myofibroblasts are cells that mainly differentiate from fibroblasts to contract wound edges and secrete new matrix (Gabbiani, 2003; Moulin et al., 1999). Matrix reconstruction, angiogenesis and reepithelialization are three mechanisms that need well organized interactions between different cell populations to correctly take place. It is accepted that communication between all these cells occurs via cytokines and growth factors secretion into the extracellular environment. However, the exact means of secretion is unclear. Numerous factors can be directly secreted into the extracellular fluid but it has also been demonstrated that membrane vesicles can represent a mechanism for regulating interactions between cells (Taraboletti et al., 2002; Taverna et al., 2003). Furthermore, even though the production of MP

by endothelial cells has been documented (Taraboletti et al., 2002), the production of MP by myofibroblast and fibroblast is still unknown.

In this study, we have shown that myofibroblasts isolated from skin granulation tissue produced a high quantity of MP in the presence of serum or plasma. In contrast, fibroblasts or myofibroblasts isolated from hypertrophic scars did not. The production of MP depended on the addition of a >30kDa-protein present in the body fluids, and was not found to be related to the induction of apoptosis in the cells. The MP produced by myofibroblasts contained VEGF and FGF2 and modulate cell growth. We thus postulate that MP production by myofibroblasts could modulate (myo)fibroblast growth and angiogenesis during the process of normal healing.

MATERIALS AND METHODS

Cell culture The human skin primary cells used in these experiments were dermal fibroblasts (Fb), normal wound (WMyo) and hypertrophic scar myofibroblasts (HMyo). WMyo from normal human granulation tissue were obtained, as previously described (Germain et al., 1994), from implants inserted subcutaneously into the arms of 20 to 40 year-old volunteers. HMyo were isolated from tissue obtained after corrective surgeries in clinically clear-cut cases of human hypertrophic scarring. Fb were isolated from human skin biopsies obtained during the myofibroblast implant surgeries (Rompré et al., 1990). Microvascular endothelial cells (EC) were isolated from the dermis as described in Rochon et al (Rochon et al., 2001). After isolation, cells were analyzed to evaluate their purity and phenotype using immunohistochemistry techniques. The numbers indicated after each population refer to the numbers that were assigned to the individuals from which the cells were isolated.

Fb and HMyo were cultured in the Dulbecco-Vogt modification of Eagle's medium (DMEM; Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL), 100U/ml penicillin, and 25μg/ml gentamicin (Sigma. St. Louis, MO, USA). WMyo were cultured under similar conditions but required addition of 20% FBS for better growth. EC were cultured in EGM-2MV (Lonza:Clonetics, San Diego, CA, USA) on gelatin-coated flask. These

cells were grown at 37ºC in a humidified incubator with 8% CO_2. Preconfluent cell cultures between passages 3 and 6 were used in this study. All specimens were obtained after the informed consent of donors according to the principles expressed in the Declaration of Helsinki.

For all experiments, the results were confirmed for at least three separate cell populations.

Human serum and plasma preparation

Pools of human serum and plasma were obtained from 15 healthy donors according to the principles expressed in the Declaration of Helsinki. Two tubes (7 ml each) (one heparinized and one not) of blood were taken from each volunteer. After centrifugation, supernatants were pooled, aliquoted and frozen at –80ºC. Fetal bovine serum (FBS) (Hyclone, Logan, UT, USA) was also tested.

Cell treatment and MP detection

Preconfluent cells were washed twice with DMEM and then, stimulated with DMEM with or without human serum or plasma, or FBS. Recombinant human PDGF (Sigma), TGFß (R&D, Minneapolis, MN), FGF2 (Lonza:Clonetics), bovine serum albumin (BSA) (Sigma), immunoglobulin G (IgG) (monoclonal anti-epithelial keratin, AE2 clone, ICN, Costa Mesa, CA, USA) were also used. After 24h of culture, both floating and trypsinized cells were pooled, fixed in cold 70% ethanol and kept at –20ºC. Flow cytometry with propidium iodide incorporation were used as previously described (Moulin et al., 2004) to routinely quantify MP production and discriminate between cells and MP. Results were expressed as a percentage of MP of the total (15 000) detected events (cells and MP).

Membrane vesicle isolation from cell-conditioned medium

MP were purified from 48h-20% FBS conditioned medium of confluent cells. Similar volume of non-conditioned medium was prepared simultaneously as control (named FBS). Briefly, medium was centrifuged at 300 x g for 10 min at 4ºC to remove cells and large debris. The supernatant were centrifuged at 21 000 x g for 20 min at 4ºC. Pelleted MP were washed with PBS and further centrifuged at 21 000xg, 20 min two times before being resuspended in PBS and lastly stored at

4ºC until subsequent in vitro use. The vesicles were quantified by measuring vesicle-associated proteins, using the microBCA assay (Fisher, Nepean, ON, Canada) with BSA as the standard.

Biochemical analysis of the serum

Serum was subjected to one of the following treatments before being tested on cells as described above. Heat treatment:10 ml DMEM containing 10% serum (or control DMEM without serum) was heated in a water bath at 86^0C for 40 minutes and then quickly cooled down in ice. Trypsin: 2 mg of trypsin (Intergen, Toronto, ON, Canada) was added to 2 ml of serum to produce a final concentration of 1 mg/ml. The media were incubated at 37^0C for 60 minutes with the proteolysis terminated with 2 mg soybean trypsin inhibitor (Sigma). Control was similarly obtained except that trypsin was added at the same time as the soybean inhibitor. Molecular sieve: 10ml DMEM containing 10% serum or plasma was centrifuged in a Vivaspin 15R (5 000, 10 000 or 30 000 MWCO) (VWR, Mississauga, ON, Canada) at 4ºC. When each solution was concentrated 10 times, each filtrate was separately stored and resulting filtered solution was further diluted with 10 ml of DMEM without serum or plasma, and centrifuged to concentrate another 10 times. DMEM was added to the remaining solution to obtain a 10 ml final volume.

Determination of cell apoptosis

Cell apoptosis was evaluated using AnnexinV/PI binding (R&D, Minneapolis, MN, USA) and TUNEL assay (R&D) as described in (Larochelle et al., 2004). Caspase-3 activity measurement was performed using the Apo Alert Caspase 3 assay kit (Clontech, Palo Alto, CA, USA). Fas antibody (Upstate, NY, USA) treatment (50ng/ml in presence of 5μg/ml cycloheximide (Sigma)) was used as a positive control.

Characterization of MP phenotype

The presence of α2-integrin protein on membrane of cells and MP was analyzed using flow cytometry on freshly trypsinized cells or MP isolated from the same cells using α2-integrin antibodies directly coupled with FITC as described in (Moulin et al., 2001). Annexin V binding was used to numerate phosphatidylserin-expressing MP as described in (Larochelle et al., 2004). VEGF and FGF2 were quantified using ELISA according to the manufacturer protocols (Peprotech, New Jersey, USA).

Cell growth stimulation using MP

The day before treatment, cells were plated at a density 15 000 (Wmyo or Fb) or 25 000 (EC) cells/well in 12-well plates (BD Biosciences, Mississauga, ON, Canada). After 24 hours, 3 wells were trypsinized to evaluate the exact number of cells in each well and other wells containing cells were then treated with MP isolated from Wmyo culture (5 and 10 μg protein/ml) in basal medium containing 0.5% FBS for 6 days before being trypsinized and counted using a Coulter® counter. Controls were performed using 100 pg/ml FGF2 (Wmyo or Fb) or EBM2 with 5% FBS (EC). Results were expressed in percentage of the cell number obtained after 24 hours of seeding.

Statistical analysis

Statistical differences were computed by analysis of variance followed by the unpaired Student's *t*-test or non-parametric SNK test (Scherrer, 1984). The results were considered significant when the *p* value was less than 0.05. All data presented are the means (errors bars: ±Standard-Deviation).

RESULTS

To determine the capacity of cells to produce MP, primary human Wmyo or Hmyo, and Fb populations were cultured in medium with and without human plasma, human serum, or bovine serum. Percentage of MP vs. total events (MP and cells) was evaluated and results were expressed as a ratio between MP obtained in presence of FBS and MP obtained in absence of FBS. The addition of 20% FBS in culture medium induced an increased production of MP for most of the Wmyo populations tested (Figure 1A). Although a few cell populations tested showed an absence of response to serum, an average increase of the ratio of 159.4% ±33.9 was obtained in the presence of 20% FBS (figure 1B). In contrast, Hmyo and Fb populations showed a decrease in the MP production observed for all populations cultured in the presence of serum when compared to cells cultured in DMEM alone (Figures 1A and B). The production of MP by Wmyo was found to be significantly increased compared to other cells whereas no difference was observed between Fb and Hmyo (Figure 1B). Isolation of MP from cell supernatants using a centrifugation technique followed by analysis of proteins showed similar results concerning the

production of MP by Wmyo, Hmyo or Fb. A significant increase in MP generation by Wmyo in presence of 20% FBS was observed in comparison with other cells (Figure 1C).

The action of serum was found to be concentration-dependant (Figure 2A) and independent of the origin of fluids (human or bovine) (Figure 2B). Human plasma induced similar results (data not shown).

Characterization of molecules affecting MP production

To characterize the biochemical nature of mediator(s) present(s) in serum or plasma responsible for the increased production of MP observed in Wmyo, several analysis were performed.

Treatment of serum with heat or trypsin impeded the serum-induced increase of MP production by Wmyo (Figure 3A). Indeed, the production of MP was not increased when heat-treated or trypsin-treated serums were used in contrast to the use of untreated serum. Furthermore, sieving of the molecules present in the serum, using a low-binding filter that allows extracting molecules with a molecular mass smaller than 10 kDa, did not affect the increased production of MP detected after serum treatment. Similarly, Wmyo cultured in the presence of serum sieved through a filter to eliminate molecules with a molecular weight smaller than 30 kDa was found to produce similar amount of MP when compared to non-sieved serum (Figure 3B). Results were similar if human or bovine sera were used (data not shown).

The action of several known proteins was also investigated on the various cell populations tested in this study. These molecules were chosen because of their quantitative importance in serum (albumin, immunoglobulin) (Anderson and Anderson, 2002), of their role in wound healing process (PDGF, TGFß) (Moulin, 1995) or in the induction of MP production (FGF2) (Taraboletti et al., 2002). The concentrations tested in this study were chosen to be in the same order of magnitude as that demonstrated in normal human serum: Albumin 35-50g/l (Anderson and Anderson, 2002); Immunoglobulin G: 0.7-4 g/l; PDGF: 17.5±3.1 ng/ml (Bowen-Pope et al., 1984); TGFß: 0.3-52 ng/ml (Rizzo et al., 1999); FGF2: 1.54±1.98 pg/ml (Larsson et al., 2002). None of the five proteins tested were found to modulate the number of MP detected after treating Wmyo (data not shown).

Apoptosis

As apoptosis is the most known mechanism at the origin of MP production, the apoptotic rate of WMyo after FBS treatment was investigated. The use of three different methods to detect apoptosis demonstrated that culture of WMyo in the presence of FBS was not found to significantly induce apoptosis in comparison to the culture in the absence of FBS (Figure 4). In contrast, Fas antibody treatment induced a high apoptotic level in these same Wmyo cells.

Characterization of MP produced by Wmyo

Since MP are vesicles derived from the plasma membrane of the original cells (Freyssinet, 2003), we validated this finding by evaluating the presence of a plasma membrane antigen: α2-integrin on the surface of the MP produced by the WMyo cells tested. As shown in Figure 5A, the presence of α2-integrin antigens was detected in plasma membranes of Wmyo and in the MP produced by these cells.

Another phenomenon of the MP is that their formation is also associated with the loss of plasma membrane asymmetry (Zwaal and Schroit, 1997). We have analyzed this parameter using annexin V binding of the outer leaflet phosphatidylserin and found that almost all of MP produced can bind annexin V (Figure 5B). The presence of these two parameters confirms that particles produced by WMyo were indeed MP as described in the literature.

VEGF and FGF2 presence in MP has been assayed since these two molecules are potent cytokines involved in cell interactions during wound healing. MP from WMyo contained VEGF (0.66±0.10 pg/μg protein (N=3 cell populations)) and FGF2 (15.51±2.52 pg/μg protein (N=3 cell populations)), whereas these two growth factors were undetectable in non-conditioned medium (Figure 5C).

Effect of MP on cell growth

To demonstrate the potential role of MP during wound healing, Wmyo, Fb and EC were treated for 6 days with two MP concentrations isolated from Wmyo. Figure 6 shows that MP significantly increased the number of cells of the three population types when 5 and 10 μg/ml of

total MP proteins were used suggesting that MP can influence cell number during wound healing. Stimulation with the higher dose of MP induced a similar growth than when 100pg/ml FGF2 (Fb and Wmyo) or EBM2 with 5%FBS (EC) were used. Cells stimulated with non-conditioned medium preparation didn't stimulate any cell growth.

DISCUSSION

During wound healing, numerous cell populations interact to orchestrate each phase of this process. When cells reconstruct new tissue, Wmyo and EC are the most important cells that are involved in the granulation tissue phase. For this to correctly take place, an intricate communication between cells needs to take place for angiogenesis and proper matrix formation. In the past, this communication was thought to be exclusively due to direct secretion of factors into extracellular space. Since the discovery that MP are not only the dusts of death cells but also mediators between cells, numerous authors have hypothesized a role of MP during wound healing. To this end, angiogenesis has been demonstrated to be modulated by MP produced by platelets or EC themselves (Taraboletti et al., 2002). In the present study, we have demonstrated that Wmyo, but not Hmyo or Fb, can also produce MP which stimulate in vitro EC growth, and thus potently in vivo angiogenesis. The MP produced by Wmyo also stimulated Wmyo and Fb cell growth, enhancing the number of matrix-producing mesenchymal cells at the wound site. Presence of VEGF and FGF2 into MP are consistent with the documented presence of these two factors during healing (Shukla et al., 1998). VEGF is a strong angiogenic factor which is involved in EC growth as well as in capillary formation (Hughes, 2008), whereas FGF2 stimulates the growth of Fb and EC (Gospodarowicz, 1991). Furthermore, the presence of FGF2 in MP has already been described as a mode for the cells to secrete this growth factor which has no conventional secretory signal sequence (Taverna et al., 2003). Production of MP by Wmyo can thus be described as a general means by which Wmyo can interact with granulation tissue cells.

Circulating MP and their role during cardiovascular or immunological diseases have been extensively evaluated (Distler et al., 2006) (Boulanger et al., 2006) due to their easy harvest from blood. In these studies, MP originated from circulating cells (platelets or blood cells) or from

vessel walls (EC and smooth muscle cells). Other studies have demonstrated that cancer cells can generate MP, stimulating cellular invasion (Graves et al., 2004; Lima et al., 2009). However, even if often postulated, few studies have demonstrated that MP produced by normal cells can play a physiological role during a non-pathological process. Production of MP by Wmyo associated with the low production of MP by Hmyo suggests that this mode of communication between cells is more important during the formation of the granulation tissue where numerous mechanisms, such as angiogenesis, cell migration or differentiation take place. Hypertrophic scar tissues are considered to be in a more stable state than wounds. The cells continue to produce matrix but generate little angiogenesis (Niessen et al., 1999). To our knowledge, this is one of the first studies demonstrating the role of MP production by local cells during a normal (i.e. non pathological) physiological phenomenon.

In culturing nucleated cells, levels of MP released into the supernatant have been correlated with the degree of apoptosis (Freyssinet, 2003). Apoptosis is therefore believed to constitute the main mechanism at the origin of the MP production. We have however demonstrated that the MP production by Wmyo is not linked to apoptotic induction of these cells as also demonstrated by few other groups using smooth muscle cells or EC (Jimenez et al., 2003; Llorente-Cortes et al., 2004; Taraboletti et al., 2002). In these studies, the MP production is triggered by FGF2, TNF or lipoproteins. We have found that serum or plasma can also stimulate MP production. In vivo, the cell environment is made of matrix and fluids containing secretions produced by adjacent cells or carried from the blood. Plasma is the body fluid at the origin of the extracellular fluid, while serum is only present in the tissues when a wound appears. Both of these body fluids are constituted of a large number of substances with very different concentrations: from 35-50 mg/ml for serum albumin to 0-5 pg/ml for interleukin-6 (Anderson and Anderson, 2002). Addition of serum in culture medium inhibits apoptosis and stimulates growth of fibroblasts (Chen et al., 2003). However, serum can also induce the production of MP by Wmyo, as our study demonstrates. To characterize the biochemical nature of mediator(s) present(s) in serum or plasma and capable of increasing MP production by Wmyo, we have performed several analysis and shown that the observed effect of these body fluids on the MP production was at least linked to thermally sensitive protein(s) with a molecular mass greater than 30 000D. This effect was not

due to TGFß, PDGF, FGF2, albumin or immunoglobulins as these molecules were not found to have any effect on this response.

This study shows differences in MP production between myofibroblasts and fibroblasts in response to normal body fluids. During normal wound healing, differentiation of fibroblasts to normal myofibroblasts induces a stronger cell sensitivity to one or more body fluid proteins. Production of MP in response to these signals could transfer bioactive molecules such as growth factors from myofibroblasts to other cells present into the wound, and serve as subcellular vectors to propagate signals and to participate to the orchestrated mechanisms of the healing process.

ACKNOWLEDGEMENTS

The authors acknowledge Charles J. Roberge for critical review and Sébastien Larochelle for his collaboration with this study. This study was supported by the Canadian Institutes of Health Research. V.M. and H.M. were recipients of scholarship from Fonds de la recherche en Santé du Québec.

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FIGURE LEGEND

Figure 1: **Normal wound myofibroblasts (Wmyo) produced more microparticles (MP) than hypertrophic scar myofibroblasts (Hmyo) or fibroblasts (Fb) in presence of 20% FBS.** A and B: Evaluation of MP presence using flow cytometry. Ratio present the percentage of MP produced by cells cultured with 20 % FBS in comparison with cells cultured without FBS. A: independent results of 9 populations of Wmyo, 6 populations of Hmyo and 8 populations of Fb. B: Mean of results obtained in A. A SNK statistical test to compare the three populations (*: $p<0.01$ vs. other populations) and a t-test comparing each population with or without FBS ($:$p<0.05$ vs. 100%±1 standard deviation) have been performed; C: Evaluation of MP recovered from cell-conditioned medium from 10^6 cells using protein assay. Similar volume of non-conditioned medium was simultaneously prepared as control and was found analogous to zero. SNK test: *: $p<0.01$ vs. other populations.

Figure 2: MP production was dependant on FBS concentration and was independent of the serum origin. A: MP percentage of the total events using flow cytometry obtained from three different populations of Wmyo in presence of several concentrations of FBS. B: MP percentage in conditioned cultures containing 10% human serum.

Figure 3: **Serum action on MP production was dependant of a thermolabile protein with a molecular weight >30kDa.** A: MP percentage of the total events using flux cytometry obtained from Wmyo-conditioned culture after 48h treatment with serum being subjected to one of the following treatments: heat, trypsin, and 5kDa cut-off molecular sieve. Representative results of experiments performed on three different populations of Wmyo. B: MP percentage on Wmyo culture with human serum obtained after a 10 or 30 kDa cut-off molecular sieving treatment. ND: not determined.

Figure 4: MP production by Wmyo was not linked to cell apoptosis . A: Activation of caspase 3 after treatment with 0 and 10% FBS or Fas antibodies. B: Annexin V staining of Wmyo after similar treatment with 0 and 10% human serum (HS) or Fas antibodies. C: TUNEL assay on Wmyo treated with 10%FBS (left) or Fas antibodies (right).

Figure 5: **MP contained α2-integrin, VEGF, FGF2 and outer leaflet phosphatidylserin.** A: Flow cytometry analysis of the presence of α2-integrin on the membrane of Wmyo (left) and MP produced by the same cells (right). The bold line corresponds to a staining with specific antibodies whereas the thin line corresponds to same staining without primary antibodies. B: Annexin V binding of MP isolated from Wmyo conditioned media. C: Results of VEGF and FGF2 detected by ELISA. Horizontal line indicates the mean of three different population results. Analysis of a non-conditioned medium preparation indicated that no VEGF or FGF2 was present in the control (*: $p<0.05$ vs. control).

Figure 6: MP addition in culture medium induced cell growth. EC (A), WMyo (B) and Fb (C) growth after a 6-day treatment with two concentrations of MP isolated from Wmyo conditioned media or 100pg/ml FGF2 (Fb and Wmyo) or EBM2+5%FBS (EC). Representative results of experiments performed on 3 different populations (*: $p<0.05$ vs. control).