Isolation of Rat Lung Mast Cells for Purposes of One-Week Cultivation Using Novel Percoll Variant Percoll PLUS

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Summary

Prolonged cultivation of separated rat lung mast cells (**LMC**) *in vitro* is necessary to better investigate a possible role of LMC in different time of model tissue remodeling induced by hypoxia. Consequently, rat lung mast cells (**LMC**) were separated here using protocol including an improved proteolytic extraction and two subsequent density gradient separations on Ficoll-Paque PLUS and a new generation of Percoll, i.e. Percoll PLUS. Instead of usual isotonic stock Percoll solution, an alternative asymptotically

isotonic stock solution was more successful in our density separation of LMC on Percoll PLUS. Separated cells were cultivated six days in media including stem cell factor, interleukins 3 and 6, and one of two alternative mixtures of antibiotics. These cultivations occurred without any contamination and with only low frequent changes in cell size and morphology. Model co-cultivation of two allogenic fractions of LMC caused frequent and sometimes rapid and considerable changes in cell morphology and size. In contrast to these observations no or rare morphological changes were found after cultivation under hypoxic conditions.

Short title: Isolation of Lung Mast Cells for Purposes of Cultivation

Key words

Hypoxia, lung mast cells, Percoll, c-kit, progenitor, secretion.

Abbreviations

FPP – Ficoll Paque PLUS; IL3, IL6 – interleukins 3 and 6, respectively; LF - lung fragments; LMC - lung mast cells; MC - mast cells; SCF - stem cell factor.

Introduction

Hypoxia-induced pulmonary hypertension accompanies several serious lung diseases, and is also involved in some cardio-vascular complications (Herget and Ježek 1989). Lung mast cells (**LMC**) participate in the mechanisms of vascular tissue remodeling activated by model hypoxia effects (Vajner *et al.* 2006, Maxová *et al.* 2008). Immediate and early responses of LMC to hypoxia are triggered with competent

receptors, adhesive molecules and oxygen/superoxide sensors (Manalo *et al.* 2005, Bell *et al.* 2007, Theoharides *et al*. 2007, Brown and Nurse 2008).

Mast cells (**MC**) can be prepared from pluripotent progenitors (**PMC**) using long-term cultivation lasting at least seven weeks (Holm *et al.* 2008). Such cultivations generate viable and well proliferating PMC. PMC are usually well adapted to *in vitro* conditions than for instance LMC. On the other hand, such type of preparation arises risk of *in vitro* artifacts. This is the reason, why LMC are still proteolytically extracted from tissues in some experiments (Holt *et al.* 1985, de Paulis *et al.* 2001, Cruse *et al.* 2005, Kim *et al.* 2005, Maxová *et al.* 2008) in spite of difficulties accompanying tissue extraction and low-yield separation (Holm *et al.* 2008). Interleukins 3, 6 (**IL3**, **IL6**) and stem cell factor (**SCF**) has been considered as cytokines necessary for MC cultivations in the last ten years (Andersen *et al.* 2008).

In this paper, we modified previously used separation of rat LMC (Maxová *et al.* 2008) for purposes of at least six-day cultivation. In addition, we utilized a new, yet very rarely employed more stable version of the cell separation medium Percoll, i.e. Percoll PLUS (representing unachievable term via PUBMED in time of submission of this paper) substituting usual Percoll dilution by asymptotically isotonic dilution. Percoll PLUS was used individually or together with Ficoll Paque PLUS, which diminishes side adherences worsening cell purity. In accordance with literature data (Wu *et al.* 2008) we found predominantly unmaturated LMC among the separated cells exhibiting c-kit positivity. This unmaturated stage is possibly a reason why frequent c-kit positive cells of fibroblast-like phenotype can be observed in response to model allogenic stimulation after cultivations lasting more than three days. Our experiments, described here, represent the starting point to model/experimental *in vitro* cultivations.

Such cultivations lasting meanwhile from half hour to three-day period of cultivation would follow three-day relaxation period diminishing consequences of necessary brutal tissue extraction of LMC.

Methods

Chemicals and equipment. Some devices, media, sera, chemicals and specific pathogen-free rats Wistar (representing allogenic individuals) necessary for our work were described in the previous paper (Maxová et al. 2008). RBL-2H3 cell line was obtained from ATCC-LGC (United Kingdom). Newly applied antibiotic Amphotericin B, Red blood cell lysing buffer, Corning filters 0.2um and chemiluminiscent peroxidase substrate 3 were delivered by Sigma-Aldrich (Missouri). Ampicillin of Biotika (Slovakia) origin was also used in our cultivation. New almost inert Percoll variant Percoll PLUS and another cell separation medium Ficoll Paque PLUS (**FPP**) were obtained from GE Health Care (Sweden). All essential cytokines, i.e. IL3, IL6 and SCF were purchased from Peprotech (New Jersey) or Prospec (Israel). Plates and Labteks (Thermo Fisher Scientific, Denmark) were sometimes placed into incubator chamber of two-liter volume (Billups-Rothenberg inc., California), which keeps hypoxic environment. Contaminating bacteria and spongi were identified by biochemical tests delivered by API Bio Mérieux (France). Peroxidase chemiluminiscent staining was developed in Kodak Image Station (Kodak, New York).

Media for LMC separation and cultivation. Media R0.5 R2, R5 contained 99.2%, 97.7 %, 94.7% of RPMI 1640 (with 20mM HEPES) and 0.5%, 2% or 5% of fetal calf serum (**FCS**), respectively, together and 0.3% of gentamycin solution. Medium R5_2E and R0.5_2E contained 99% R5 or R0.5, respectively, and 1% 200 mM EDTA pH 7.5.

In accordance with Biotech protocol for model cell line RBL-2H3 (cf. www.biotech.inst. unige.it/clbd/cl5348) all LMC fractions were also cultivated in special medium with and 0.3% gentamycin and 10% FCS (primary tissue culture medium, **PTC medium**) or 10% additional RPMI (proteinase incubation medium, **PI medium**). To prepare cultivation medium we added to PTC medium 3% amphotericin B (fungison) solution, 0.03% of 1% ampicilin solution and cytokines SCF, IL3 and IL6, all in the same final concentrations 20 ng/ml (medium TCa3 in the following text; cf. Arinobu *et al.* 2005).

Schemes of separation. For schemes of our starting and final protocols see Table 1. For unchanged procedures see the previous paper (Maxová *et al.* 2008). More detailed protocol can be received by e-mail.

Table 1. Schemes of LMC separation

Original protocol[a] (Maxová et al. 2008)

lung perfusion → *segmentation to lung fragments (LF)* → **collection of LF samples** → *processing LF with trypsin* → proteolytical extraction → *initial clumps of extracted cells were removed with help of sieve* → **Percoll** → *two-step washing cascade (200 g, 2 x10 min in R5)*

New protocol[a,b]

lung perfusion → *segmentation to LF* → **soaking procedure** → *processing LF with trypsin* → proteolytical extraction 1 → sample collection 1 → proteolytical extraction 2 → sample collection 2 → *initial clumps of extracted cells were removed with help of sedimentation (gravity sedimentation 5-10 min or centrifugation puls 200g, 5s)*
→ **modified Ficoll Paque PLUS (mFPP)**[c] → **Percoll PLUS**[c] → *pellet*
→ **Percoll PLUS**[d] → *pellet*
pellet → **erythrocyte lysis** → *washing with EDTA (200 g, 10 min in R5 E2)*
→ *two-step washing cascade (200 g, 2 x10 min in R5)*

[a]Original protocol represents LMC separation for purposes of short experimental cultivations lasting usually one day (cf. sections 4.1 and 4.2). Italic – unchanged procedures; italic underlined – different procedures mentioned only here; underlined - modifications are mentioned in the text; bold - new or markedly different procedures described or explained in the text.
[b]For description of new procedures and modifications see sections 2.4-2.8.
[c,d]An alternative procedure including either subsequently employed mFPP and Percoll PLUS or solely Percoll PLUS, respectively.

*Soaking procedure and proteolytical extraction (**PE**).* Soaking procedure comprised repeating steps including cutting of starting or still floating lung fragments (**LF**) and collection of LF pelleted by centrifugation pulses (1s, 400 g). Soaked LF were two times incubated 40 minutes in 2-3ml aliquots of proteinase mixture completed by delayed addition of 0.4-0.6 ml DNAase after 20 minutes of each PE (proteinase mixture: 5 mg collagenase, 5 mg hyalurinidase and 0.25 ml elastase in 16 ml of PI; DNAase solution: 1 mg DNAse were solved in 4 ml of PI; both solutions were filtered). Both PE included two one-minute circular shakings occurring after each twenty-minute lasting period. The transfer of released cells to R5 terminated both PE (centrifugation 15 min, 300 g).

Separation of LMC on discontinuous gradients of Percoll PLUS and modified FPP. Instead of usual isotonic Percoll (Maxová *et al.* 2008), we prepared **asymptotically isotonic Percoll PLUS** (**AIPP** contained 22.73 ml of Percoll PLUS and 2.27 ml 1.5M NaCl, cf. first section of Results) by mixing with Percoll PLUS with R0.5_2E to perform 12 ml 35%, 75% and 90%. Cell suspensions in R5 were applied to preformed six gradients and centrifuged for 20 minutes (400 g, in accordance with GE Health Care handbook Percoll_18111569AD). Separation of LMC was performed individually or after separation on modified FPP. In latter case, 0.1% volume of FCS was added to FPP immediately before separation and resulting solution was filtered (filter cut off limit 0.2 um). This filtered solution was then used in separation of LMC fraction accumulating in a pellet (250 g, 15 minutes, 18-25^{o}C).

Erythrocyte lysis. Supernatants were thoroughly splashed away after Percoll centrifugation. Pelleted cells were resuspended in 0.7 ml of red blood cell (erythrocyte) lysing buffer, incubated one minute in flow box and diluted by 2.8 ml of cold R5_2E.

Prevention of cell clumping and diminishing of interactions between bacteria and separated cells. To prevent accumulative formation of cell clumps we used: i) FCS in all media (FCS was also present in concentration 0.5% in medium R0.5_2E diluting Percoll PLUS), ii) cooled centrifuges and probes in ice, iii) cell suspension without initial clumps (discarded after proteolytical extraction). The interactions of separated cells with bacteria were blocked by: i) cleavage of interacting molecules during proteolytical extraction or ii) inhibition of some such interactions in presence of 2 mM EDTA during subsequent cell separation. The effect of EDTA concerns first of all widely spread interactions between i) lectins or integrins and ii) bacterial cell surface molecules (Smith 1997, Hosoi *et al.* 1998, Zelensky and Gready 2005, Eto *et al.* 2007, Yu *et al.* 2007).

Cultivation, hypoxic cultivation and transfer of LMC fraction. Majority of *in vitro* cultivations occurred in sterile LabTek using medium TCa3. Three-day-lasting pre-cultivations preceding hypoxia exposure were carried out to minimize the most frequent cell-death events initiated during the starting cell separation (cf. Bischoff *et al.* 1999). During hypoxic experiments in vitro, cells were placed into modulator incubator chamber with 3% O_2, 5% CO_2 and 92% N_2. Gas equilibration was performed fifteen minutes by flow rate 2 liters of gas per minute. All cells of LMC tissue culture were de-attached from well surface after ten-minute incubation with 0.2% trypsin in PBS (similarly to cell line RBL-2H3, http:// www.atcc.org).

Microbiological analysis. Samples (media, washings or cell suspensions) were transferred to nine times higher volume of amplifying thiglycolate buoyant Dulab and incubated 18-24 hours at temperature 35^{o}C in aerobic environment. The resulting solutions were transferred to the blood, End´s and Saboraud´s agars and incubated 36

hours in the same temperature. Macroscopical and microscopical morphologies and standard biochemical tests were used to identify contingent colonies of microbes.

Cytological analysis. Toluidine blue, peroxidase-DAB visualization with c-kit (CD117) and hematoxylin counterstaining enabled us to check cell phenotype and maturation stage of methanol-fixed LMC (Churukian and Schenk 1982, Avivi *et al.* 1994). At least ten different cell numbers obtained in five defined sites (microsamples) of at least two Labtek wells formed minimal data set.

Detection of MMP13 in exosomes of RBL-2H3. Exosomes present in two times filtered (filter cut off limit 0.2 μm) medium (pre-sedimented by two subsequent centrifugations 500g, 10 minutes) from six-day tissue culture of 10^7 RBL-2H3 cells origin were sedimented by ultracentrifugation (100 000 g, 2 hours). Vortexed exosomes were solved in sample buffer for SDS electrophoresis and heated up five minutes in boiling bath. Resulting solution was then diluted to achieve exosome amounts corresponding to rounded off numbers of source RBL-2H3 cells. For electrophoretic and blotting procedures see Maxová *et al.* 2008. The peroxidase immunostaining of blot replica was performed with our monoclonal antibody against MMP13, peroxidase substrate 3, the corresponding Sigma protocol and light sensitive camera present in Kodak Image Station.

Statistical evaluation. All enumerations were performed on minicomputer Casio Algebra PLUS. The evaluation included programming in Basic language comprising functions enabling usage of statistic modules of given minicomputer (for formulas see Komenda 1997 and Zvárová 2001). All n6* values (mean cell numbers after 6 days) higher than limiting n3+ represented the values significantly higher than **n3*** (mean cell

numbers after 3 days) determined with help of Student´s t-test at level $p < 0.05$. For additional details see Zvárová 2001.

Results

Innovations and improvements in cell separation by Percoll. Routine procedure of current Percoll preparation was less successful, when employing it in case of Percoll PLUS. This conclusion was implicated by layer distribution of cells markedly different from the expected distribution. Since weak hypotonicity is recommended for related separation of leukocytes (280-300 mOsM) in some papers (Boyum *et al.* 2002), we prepared initial stock solution as 100% asymptotically isotonic Percoll PLUS (**AIPP**; related to maximum achievable osmolarity of Percoll PLUS) instead of usual isotonic stock solution.

Separation on AIPP derived gradients achieved sufficient agreement with expected layer distribution of cells indicating layers with: i) dead cells (35% AIPP), ii) leukocytes and fibroblasts (75% AIPP), iii) thin or no residual cell impurities (90% AIPP) and iv) mast cells and residual erythrocytes (pellet).

The quality AIPP-related gradient was also tested with help of density marker beads (**DMB**). In accordance with desired mast cell density range (1.102-1.119 g/ml, Maxová *et al.* 2008), green DMB (density 1.098 g/ml) were located about 2 cm under bottom floating on the layer 90% AIPP, whereas the red DMB (density 1.120 g/ml) were present in pellets. We did not observed any substantial differences between yields of separations, when comparing Percoll and Percoll PLUS, except for the gradual failing of separations on (old) Percoll appearing after about three-months of its usage.

Combination of modified FPP and Percoll PLUS. Two-step separation resulted in yield of about 20-60% cells of one-step Percoll fractionation (i.e. 5-25x10^4 cells per single rat lung) but substantially diminished well-observable cell adherences to probe sides located in density boundary lines and similar to beer circles. Partial diminishing of side adherences was also achieved by application of cells to Percoll PLUS gradients in R5 medium. Resulting cell suspension of LMC, uninfluenced by any corpuscular interaction, achieved 70-90% purity determined by c-kit expression after three- or six-day cultivations.

Microbiological screening. The new antibiotic mixture (**ATB3**) described in Methods enabled us even sixty-day cultivation without any contamination. The occurrence of contamination in case of current experiments was lower than 5% and included *Acinetobacter spp., Staphyloccocus epidermidis* (necessity of gloves usage during MC preparation). Nevertheless, frequent contamination can sometimes occur when adding (lung cell extracting enzyme) pig pancreatic elastase I without mixing to the PI medium (crystal of enzymes remain in bottom of stock solution).

Morphology of separated rat LMC cultivated after separation. In accordance with literature data (Wu *et al.* 2008) and photos of LMC of various species origin published on web, majority of LMC separated by our procedures exhibited un-maturated morphology (Figures 1 and 2). Some preparations of LMC included among others the cells, which looked like to contain two or several nuclei (cf. Discussion).

Surprisingly, some fibroblast-like cells were c-kit positive (Figure 2) indicating thus morphological but not phenotypic deviations. These cells and fibroblast impurities however more rapidly grow than spherical cells. Fibroblast-like cells were also observed after some one-step Percoll separations (Figure 2 b and d) and during model allogenic

reaction (Table 2). Proliferation of spherical cells in LMC tissue culture was usually stopped after 10-15 days of long-lasting cultivations, whereas the fibroblast and fibroblast-like cells still proliferated.

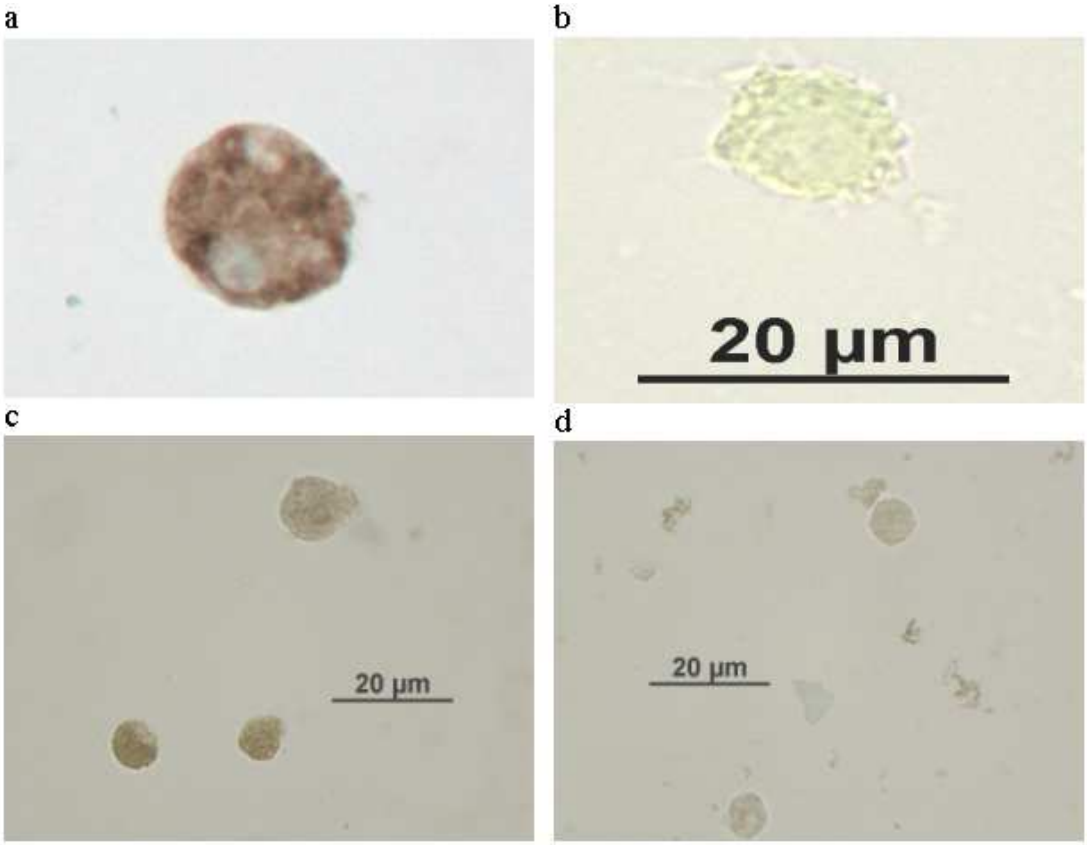

Fig. 1. Immunohistochemical detection of c-kit with LMC. a,c – c-kit-positive cells; b,d –negative controls; a,b – six day cultivation; c,d – three day cultivation.

5-30% lower fractions of fibroblast-like cells were found when comparing model three-day hypoxic cultivations with the normoxic ones. The morphological differences between hypoxic and normoxic cells were not observed when using toluidine blue staining indicating large LMC granules (Figure 2). In agreement with this fact, molecules MMP13, found in enlarged amount in LMC after hypoxia (Maxová *et al.* 2008), were detected by blotting in distinct membrane compartment of model cell line RBL-2H3, i.e. in exosomes representing descendants of small intracellular membrane vesicles invisible with light microscopy (cf. Figure 3 and Discussion).

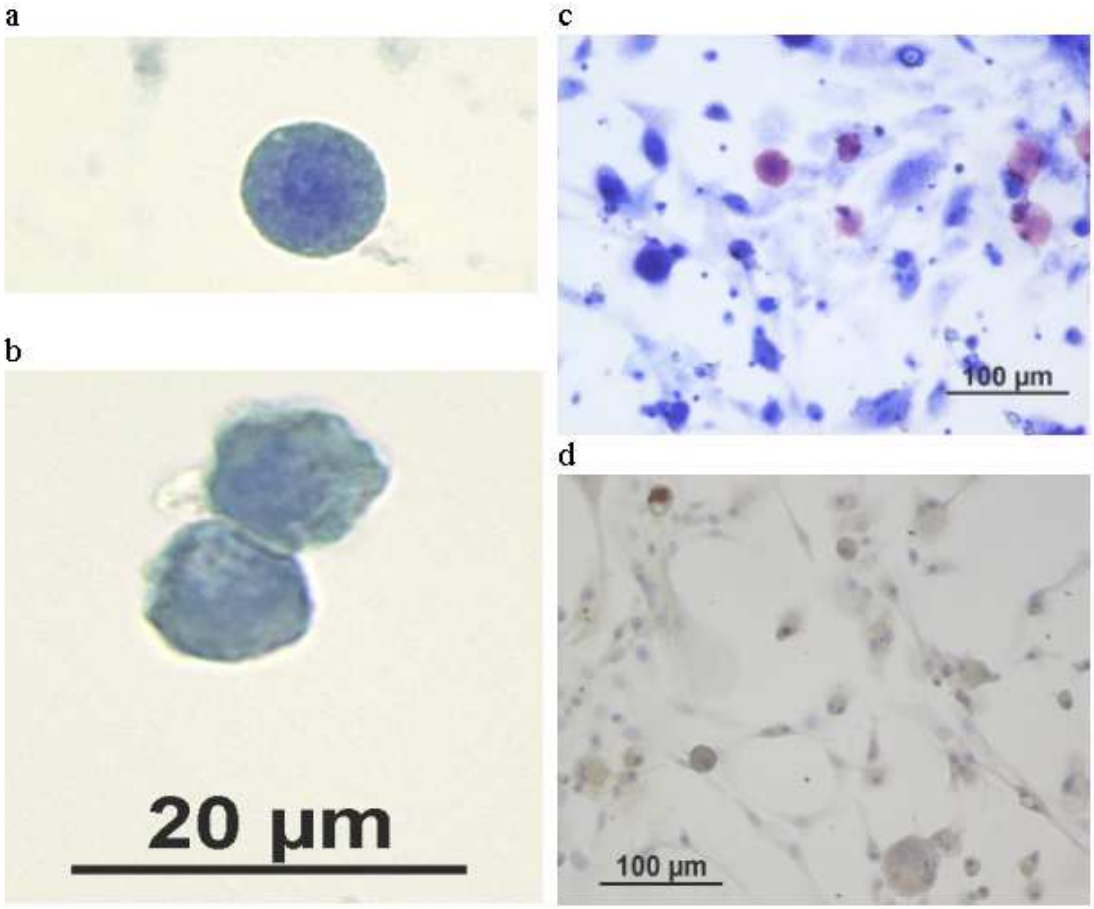

Fig. 2. Morphology of cultivated lung mast cells. a, b, c – six-day cultivation, d – three-day cultivation; a, b – comparable toluidine blue staining after hypoxic or normoxic cultivations, respectively; c, d – cells after one step separation using Percoll PLUS only; c – purple metachromatic maturated and blue unmaturated LMC; d - staining with DAB and hematoxylin indicating simultaneous presence of fibroblasts (“gray cells“) and c-kit positive fibroblast-like cells and giant LMC (“brown cells“).

Model cultivation with LMC fraction. The results presented in Table 2 demonstrate low but significant proliferation rates of LMC during three-day period meanwhile assumed for experiments, which would follow three-day relaxation period (cf. mean cell numbers n6* with enumerated limit n3+ in Table 2). The required optimal size and shape was found in the cases of proliferation rate (**PR**) lower than 0.3 divisions per day (**DPD**). Cells cultivated with our antibiotic mixture (odd rows) exhibited: i) less deviated morphology (row pairs [1,2], [3,4], [5,6], [7,8] in Table 2) than those incubated

Table 2. Model cultivation of separated cells

	experimental values and enumerated limits[a]				cell proliferation and morphology	
	n3*	**n6***	**sd_gr**	**n3+**	**div/day**[b]	**phenotype**[c]
[d]**1**	3.20	5.80	0.88	4.81	**0.286**	f-,n(l)
2	2.90	6.50	0.83	4.41	**0.388**	f-_f(l),n/l
3	4.10	10.40	1.43	6.72	**0.448**	f+,n/l_l
4	7.10	19.90	2.41	11.52	**0.496**	f++,vl
5	3.70	5.90	0.66	4.91	**0.224**	f-,n_n(l)
6	3.40	5.70	0.90	5.05	**0.248**	f(l),n(l)
7	4.90	8.90	0.88	6.52	**0.287**	f(l),n(l)_n/l
8	6.30	14.00	1.27	8.62	**0.384**	f++,vl

[a]Significant proliferation and different morphologies were observed in our differently cultivated samples. This conclusion follows from the comparison of **n3+** and **n6*** values. **n3+** - n6* values higher than n3+ are significantly higher (w = 0.05) than n3*; **n3***, **n6*** - mean numbers of cells after three and six days of cultivation, respectively; **sd_gr** – group-related standard deviations. For additional comments see last section of Results.

[b]div/day – Proliferation rate (**PR**) denoting number of cell divisions per day (PR = log(n6*/n3*)/(days x log2), where days = 6-3 = 3).

[c]cell size and morphology after 6 days of cultivation: f- - absence of cells with fibroblast phenotype (**CFP**); f(l) - low occurrence (less than 10%) of CFP; f+ - comparable numbers of normal cells and CFP; f++ - predominant occurrence of CFP; n – normal size; n(l) – normal cell size and low occurrence (less than 10%) of larger cells; n/l - comparable numbers of normal size and large cells; l – dominant occurrence of large cells; vl – majority of cells was substantially higher than the cells classified as large.

[d]rows including data: 1-4 – cultivation with SCF, IL3, IL6; rows 5-8 – in addition to the preceding cytokines we added filtered (cut off limit 0.2um) medium from 6 day RBL-2H3 cultivation to its final 10% dilution in the original cultivation medium; odd, even rows – our mixture of 3 antibiotics or "antibiotic antimycotic solution" were added to media, respectively; rows 1,2,5,6 – autologous cells; rows 3,4,7,8 allogenic cells.

with "antibiotic antimycotic solution" and ii) significantly lower cell growths ($p < 0.05$, and t-test for pairs were used here and in the following part of this section, Komenda 1997). The model samples including allogenic cells from two different Wistar rats (samples 3,4,7,8) achieved significantly higher growth ($p < 0.05$) and more deviated morphology (possibly due to allogenic reaction of residual lymphocytes) than in the

culture with cells of single rat origin (compared pairs [1,3], [2,4], [5,7], [6,8]). Samples corresponding to the pairs of table rows: [1,5], [2,6], [3,7], [4,8] differed only by addition of one ninth of filtered cultivation medium without cytokines (or related medium with "antibiotic antimycotic solution") from one-week cultivation of about 10^6 RBL-2H3 cells (in 10ml of medium) to the cultivation medium with samples 5-8. This addition of RBL-2H3 derived medium significantly diminished proliferation rates in all compared pairs of samples ($p < 0.02$) and was accompanied by the almost optimal morphology (Table 2). This effect concerned even the interesting case of model allogenic culture containing our antibiotic mixture ABT3 (cf. rows three and seven in Table 2).

Discussion

Comparison of our LMC separation with related methods. Since separation of rat LMC is poorly mentioned in literature, we compare here our method with the methods proposed for separation of human LMC. In comparison with LMC separation using direct interaction with magnetic beads (Cruse *et al.* 2005) our LMC did not contain interacting residual particles. On the other hand, the compared direct magnetic separation is excellent with respect to purity (98% of purity) and without any problems when isolating LMC specific lysates for biochemical and molecular biology purposes. In contrast to our method, elutriation appears to be more interesting with respect to short cultivations (Willheim *et al.* 1995). Hence disinfections of elutriation rotor are still carried out simply by less reliable ethanol (Binda *et al.* 2009). Nevertheless, the possibility to remove impurities of variously differentiated cells by magnetic separation (Willheim *et al*. 1995) appears to be interesting supplement to our future method

improvement. Immediate solution of such improvement however yet stagnate with less extended repertoire of necessary anti-rat antibodies.

Problems with contamination. Separation of LMC for purposes of one-week or longer cultivations is less frequently described in the literature than the other LMC separations (Cruse *et al.* 2005). The main obstacles follow from the air exchange in lung implicating a contamination risk. Among the many effects influencing sterility of cultivation, we consider here: i) effect of proteolysis and low concentrations of EDTA on cell surface interactions (see section 2.8), ii) effect of antibiotics and iii) anti-fungal and bactericidal effects of proteolysis.

Our introductory microbiological and microscopical screening concerned samples of MC separated by Percoll gradient centrifugation, which were cultivated in medium containing gentamycin (**G-samples**). Analysis of positive G-samples revealed molds and bacterial contaminations sensitive to rifampicin and vankomycin. Both these antibiotics however triggered release histamine release in some case (Nessi *et al.* 1976, Chiang *et al.* 1992, Nabe *et al.* 1999, Toyoguchi *et al.* 2000) and thus that they do not fit to our experiments with MC. Subsequently, we decided to introduce ampicillin to the proposed improved medium due to its less specific but important synergistical effect with the previously mentioned gentamycin (Gnarpe *et al.* 1976) and amphotericin B (fungison) as anti-mold agents.

Provided that we assume anti-microbial effects of proteases composing tissue extraction mixture, first pig pancreatic elastase 1 (**PPE1**) appears to be a reasonable candidate for such activity. This would be in accordance with the positive effect of PPE1 mixing on cell culture sterility described above. In addition, local (BLASTP derived) sequence similarity between PPE and anti-fungal and bactericide human

neutrophil elastase (**HNE**; Miyasaki *et al.* 1991, Newman *et al.* 2000) belongs to superior ones among vertebrate pancreatic elastases (the second order was indicated in time before the paper submission). This fact suggests marked convergent usually functionally driven changes in PPE1 sequence, though PPE1 can be well distinguished from HNE-related neutrophil elastase family using our topic PSI BLAST/Clustal W-derived phylogram (data not shown).

Proliferation and morphology of LMC. In accordance with literature majority our LMC represent unmaturated cells (Wu *et al.* 2008) enabling usually broader changes in phenotype. Rapid proliferation (proliferation rates higher than 0.35 divisions per day) was frequently accompanied by considerable changes in size (enlargement of spherical cells) and rapid changes in morphology including generation of observed fibroblast-like cells (Table 2). Such changes were predominantly observed in model mixtures of allogenic cells (of different Wistar rat origin) generating cytokines. In accordance with expression studies of model human mixed lymohocyte reaction (Kohka *et al.* 1999, Itoh *et al.* 2002) and superior expression of interleukin 18-receptor (IL18R1) in human mast cells (item GD1775/206681_at/ IL18R1 present in expression database of NIH), interleukin 18 appears to be a possible candidate for generation of the discussed fibroblast-like cells.

Fibroblast-like morphology of LMC mentioned in Results (see also Figure 2) is related to morphology of more rapidly growing rat basophilic leukemia cell line RBL-2H3, representing widely used model of mast cells. Nevertheless, addition of medium from six-day cultivation of RBL-2H3 to medium with LMC to its final concentration 10% diminished phenotypic changes (spherical cells of unchanged size; Table 2). It is a question whether nerve growth factor, interleukin 6 or tumor necrosis factor alpha

detected in supernatants of uninfluenced RBL-2H3 (Suzuki *et al.* 1998, Onose *et al.* 2008) participate in such modulation or we can assume effects of membrane particles of 60-90 nm extent called exosomes released by both mast cells and model RBL-2H3 (Skokos *et al.* 2002, Laulagnier *et al.* 2005).

LMC looking like double- or multi-nuclear cells were observed in some samples (Figure 2d). In accordance with literature data these cells contain more likely segmented nuclei than actually multiplied nuclei. Hence mast cells with segmented nuclei are indeed observed in tissue cultures and they can imitate cells with multiplied nuclei in some of their photos (Gurish *et al.* 1997, Chott *et al.* 2003).

The observation that hypoxia do not change density of large (toluidine blue positive) histamine granules of extent 800 – 1000 nm looks like to contradict to changes in MMP13 synthesis described in the previous paper (Maxová *et al.* 2008; Theoharides *et al.* 2007). This apparent contradiction can be explained by presence of MMP13 and perhaps other secreted molecules induced by hypoxia in membrane compartment distinct from large granules (cf. Results and Figure 3). Based on presented data this compartment is composed of small vesicles with diameter about 40 – 80 nm (Theoharides *et al.* 2007), representing precursors of the separated exosomes (Skokos *et al.* 2002; cf. also Methods). The investigated molecule MMP13 is otherwise important with respect to lung tissue remodeling induced by hypoxia. Hence it disrupts the quaternary organization of triple helix in the collagenase susceptible site (Weingarten and Feder 1986, Novotná and Herget, 2002) initiating thus generation of matrikines (Maxová *et al.* 2008).

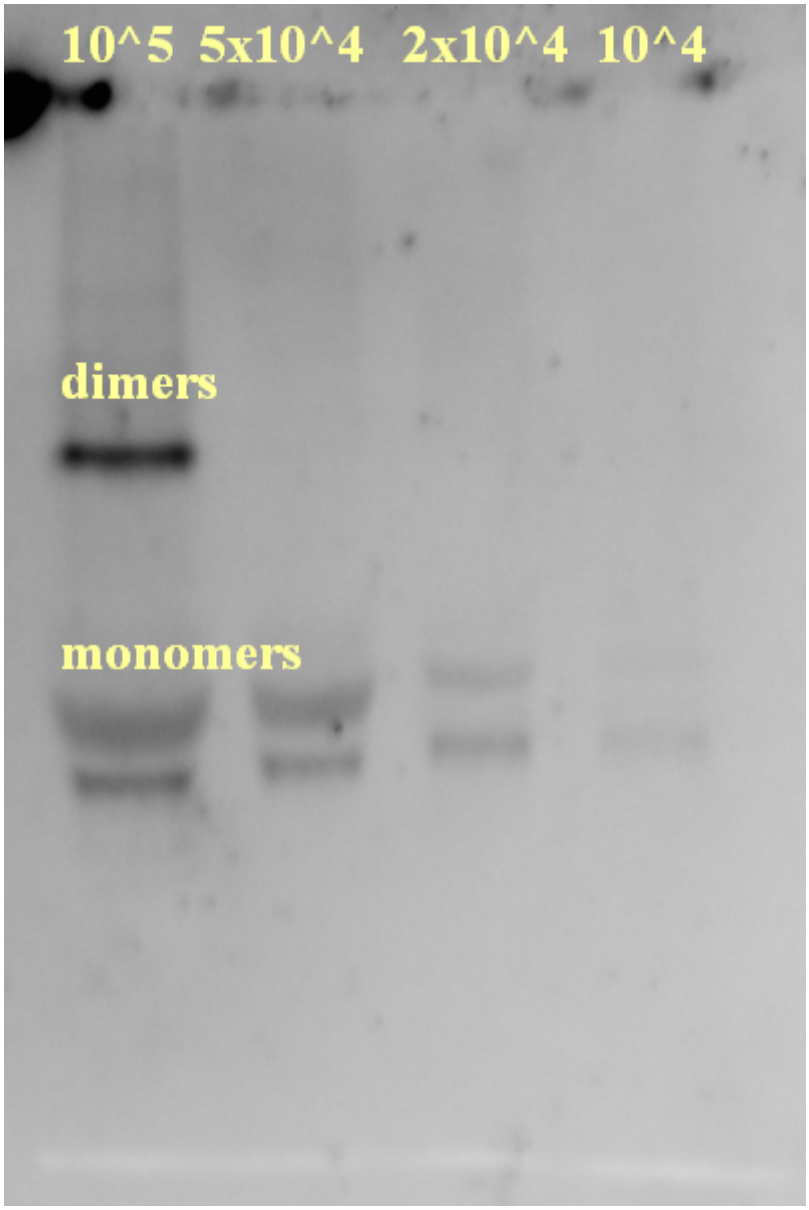

Fig. 3. Blotting detection of MMP13 in exosomes from model cell line RBL-2H3. MMP13 was still detected in minimum amount corresponding to 10^4 of source RBL-2H3 cells. We can also observe single band with MMP13 dimers (caused by low dilution of preparation with sample buffer), and double-bands containing larger pro-enzyme and smaller active form of MMP13 (cf. Novotná and Herget, 2002). For details of blotting detection see Methods.

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