

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 stages of tissue remodeling induced by hypoxia. Rat lung mast cells (LMC) were separated using a 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 for six days in media including stem cell factor, interleukins IL-3 and IL-6, and one of two alternative mixtures of antibiotics. These cultivations were performed without any contamination and with only rare changes in cell size and morphology. Model co-cultivation of two allogenic fractions of LMC often caused considerable rapid changes in cell morphology and size. In contrast to these observations no or rare morphological changes were found after cultivation under hypoxic conditions. In conclusions, we modified separation on Percoll PLUS to be widely used, altered LMC separation with respect to purposes of long-lasting cultivation and observed some model morphological changes of LMC.

Key words

Hypoxia • Lung mast cells • Percoll • C-kit • Progenitor • Secretion

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Introduction

Hypoxia-induced pulmonary hypertension accompanies several serious lung diseases, and is also involved in some cardiovascular complications (Herget and Ježek 1989). Lung mast cells (LMC) participate in the mechanisms of vascular tissue remodeling activated by hypoxia (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 better adapted to the *in vitro* conditions than LMC. On the other hand, such type of preparation increases the risk of *in vitro* artifacts. This is a 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 and 6 (IL-3, IL-6) and stem cell factor (SCF) has been considered as cytokines necessary for mast cell cultivations in the last 10 years (Andersen *et al.* 2008).

In this paper, we modified previously used separation of rat LMC (Maxová *et al.* 2008) for the purpose 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 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 the data of Wu *et al.* (2008) we found predominantly immature LMC among the separated cells exhibiting c-kit positivity. This immature 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 represent the starting point to experimental *in vitro* cultivations. Such cultivations lasting from half hour to three days would be preceded by three-day relaxation period diminishing the consequences of necessary brutal tissue extraction of LMC.

Methods

Animals, chemicals and equipment

Adult male Wistar specific pathogen-free rats (Anlab, Prague, Czech Republic) weighing 300-450 g were used in each of our twelve LMC separations (3-9 rats per experiment). Lung extractions were performed in accordance with the European Community and NIH guidelines for using experimental animals and were approved by Animal Studies Committee of our institution. RBL-2H3 cell line was obtained from ATCC-LGC (Teddington, United Kingdom). Fetal calf serum was purchased from Biochrom (Berlin, Germany). Media RPMI1640 and E-MEM both with 20m M HEPES, an antibiotic mixture “antibiotic antimycotic solution”, antibiotic amphotericin B, Red blood cell lysing buffer, Corning filters 0.2 µm and chemiluminiscent peroxidase substrate 3 were all delivered by Sigma-Aldrich (St. Louis, Missouri). The antibiotics ampicillin (Biotika,

Slovenská Lupa, Slovakia) and gentamycin (Lek Pharmaceuticals, Ljubljana, Slovenia) were also used in our cultivations. New almost inert Percoll variant Percoll PLUS and another cell separation medium Ficoll Paque PLUS (FPP) were obtained from GE Health Care (Uppsala, Sweden). All essential cytokines, i.e. IL-3, IL-6 and SCF were purchased from Peprotech (Rocky Hill, New Jersey) or Prospec (Ness-Ziona, Israel). Plates and Labteks (Thermo Fisher Scientific, Kamstrupvej, Denmark) were in some experiments placed into incubator chamber of two-liter volume (Billups-Rothenberg Inc., Del Mar, California), which keeps hypoxic environment. Contaminating bacteria and spongi were identified by biochemical tests delivered by API Bio Mérieux (Marcy l'Etoile, France). Peroxidase chemiluminiscent staining was developed in Kodak Image Station (Kodak, New York, New York).

Media for LMC separation and cultivation

Media R0.5 R2, R5 contained 99.2 %, 97.7 %, 94.7 % of RPMI 1640 (with 20 mM HEPES) and 0.5 %, 2 % or 5 % of fetal calf serum (FCS), respectively, together with gentamycin (final non-toxic concentration 120 µg/ml). 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 DSMZ protocol for cultivation of cell line RBL-2H3 (i.e. a line used as a mast cell model; cf. www.dsmz.de), primary tissue culture medium (PTC medium) contained 70 % MEM (with Earle's salts), 20 % RPMI 1640, 10 % FCS and gentamycin (final concentration 120 µg/ml). Similar proteinase incubation medium (PI medium) then included 10 % of additional RPMI instead of FCS. To prepare our cultivation medium we added amphotericin B (fungison) and ampicillin to PTC medium in order to achieve their final concentrations 7.5 µg/ml and 3 µg/ml, respectively. In addition, all the cytokines SCF, IL-3 and IL-6 were present in the same medium at final concentrations 20 ng/ml (Arinobu *et al.* 2005).

Separation and cultivation of mast cells

Schemes of separation

The schemes of our initial and final protocols are shown in Table 1. For unchanged procedures see the previous paper (Maxová *et al.* 2008).

Soaking procedure and proteolytical extraction (PE)

Soaking procedure comprised repeating steps including cutting of still floating lung fragments (LF) and

Table 1. Schemes of LMC separation.

Original protocol a (Maxová et al. 2008)	New protocol a,b
<i>lung perfusion</i> <i>segmentation to lung fragments (LF)</i> collection of LF from four rats <i>processing LF with trypsin</i> <u>proteolytical extraction</u> <u>clumps of non-digested tissue were removed using a sieve</u> performed continuous Percoll gradient <i>washing (300 g, 30 min in RPMI 1640)</i>	<i>lung perfusion</i> <i>segmentation to LF</i> soaking procedure <i>processing LF with trypsin</i> <u>proteolytical extraction 1</u> <u>sample collection 1</u> <u>proteolytical extraction 2</u> <u>sample collection 2</u> <u>clumps of extracted cells were removed with help of sedimentation, i.e. using gravity sedimentation 10 min on ice or centrifugation pulse 200 g, 5 s^e</u> modified Ficoll Paque PLUS (mFPP)^c <i>pellet</i> Percoll PLUS^c <i>pellet</i> Percoll PLUS^d <i>pellet</i> erythrocyte lysis <u>washing with EDTA (200 g, 10 min in R5 E2)</u> <u>two-step washing cascade (200 g, 2 x10 min in R5)</u>

^aOriginal protocol represents LMC separation for purposes of short experimental cultivations lasting usually one day. Italics – unchanged procedures; italics 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. ^bFor additional details see Methods. ^{c,d}An alternative procedure including either subsequently employed mFPP and Percoll PLUS or solely Percoll PLUS, respectively. ^eFor similar pulse see *Soaking procedure and proteolytical extraction*.

collection of LF pelleted by centrifugation pulses (each centrifugation pulse comprised: i) fast acceleration to 400 g, ii) keeping maximum speed for one second, iii) braked deceleration to 150 g, and iv) final deceleration without braking). Soaked LF were two times incubated for 40 min in 2-3 ml aliquots of proteinase mixture completed by delayed addition of 0.4-0.6 ml DNAase after 20 min of each PE (proteinase mixture: 5 mg collagenase, 5 mg hyaluronidase and 0.25 ml elastase in 16 ml of PI; DNAase solution: 1 mg DNAase were solved in 4 ml of PI; both solutions were filtered). Both PE included two one-minute circular shakings occurring after each 20-min 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 1.5 M NaCl, see also the 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 six probes with preformed gradients and centrifuged for 20 min (400 g, in accordance with GE Health Care handbook 18-1115-69). 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 µm). This filtered solution was then used in separation of LMC fraction accumulating in a pellet (250 g, 15 min, 18-25 °C).

Erythrocyte lysis

Supernatants were thoroughly splashed away after Percoll centrifugation. Pelleted cells were resuspended in 0.7 ml of 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 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 Greedy 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 our cultivation medium. 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 (Bischoff *et al.* 1999). During hypoxic experiments *in vitro*, cells were placed into modulator incubator chamber with 3 % O₂, 5 % CO₂ and 92 % N₂. Gas equilibration was performed for 15 min by gas flow rate of 2 l/min. All cells of LMC tissue culture were detached from well surface after 10-min incubation with 0.2 % trypsin in PBS (similarly to cell line RBL-2H3, cf. www.lgcstandards-atcc.org).

Experimental procedures and their evaluation

Microbiological analysis

Samples (media, washings or cell suspensions) were transferred to nine volumes of amplifying thioglycolate buoyant Dulab and incubated 18-24 h at temperature 35 °C in aerobic environment. The resulting solutions were transferred to the blood, End's and Sabouraud's agars and incubated 36 h 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 10 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 (presedimented by two subsequent centrifugations 500 g, 10 min) from six-day tissue culture of 10⁷ RBL-2H3 cells were sedimented by

ultracentrifugation (100 000 g, 2 h). Vortexed exosomes were solved in sample buffer for SDS electrophoresis and heated up for 5 min 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 calculations were performed on minicomputer Casio Algebra PLUS. The evaluation included programming in Basic language comprising functions which enabled us the use of implemented statistic modules of the given minicomputer (for formulas see Komenda 1997 and Zvárová 2001). Each statistical limit (n+) was determined with help of one-side Student's t-test according to the formula:

$$n+ = n^* + Q(w, df) \times S \quad (1)$$

where n^* is mean value, $Q(w, df)$ was the corresponding quantile, w denoted significance level and S is overall group-related standard deviation. Usually holds that $df = k + m - 2$ (where k and m are numbers of compared values in two sets) but here we related S to the mean value defined by single set (ten numbers) only (cf. section *Cytological analysis*). Consequently, we used more skeptical (i.e. higher limit related) statistical estimation determined by the single set derived $df = k - 1$. S was defined by the formula:

$$S^2 = \left\{ \left(\frac{1}{k} + \frac{1}{m} \right)^{0.5} \right\} \times \left\{ (k-1) \times S_1^2 + (m-1) \times S_2^2 \right\} / (k+m-2), \quad (2)$$

where S_1 and S_2 are standard deviations of the first and second sets, respectively (Zvárová 2001).

Results

Innovations and improvements in cell separation by Percoll

Currently prepared initial (apparently "isotonic" 100 % Percoll was less efficient in cell separation than that of Percoll PLUS. This conclusion was implicated by marked difference between expected and observed layer

distributions of separated cells. Since weak hypotonicity is recommended for related separation of leukocytes (280-300 mOsmol/l) by Boyum *et al.* (2002), we prepared an “asymptotically isotonic” 100 % Percoll PLUS solution (AIPP; related to maximum achievable osmolality of commercially accessible stock Percoll PLUS 30 mOsmol/kg) instead of current initial “isotonic” 100 % solution. It means that we shifted the maximum possible concentration of Percoll PLUS following from the current Percoll dilution, i.e. about 327 mOsmol/l, to the upper AIPP-related optimum value 300 mOsmol/l. In fact, this represented a simple mixing of ten volumes of Percoll PLUS and one volume of 1.5 M NaCl, instead of the corresponding nine volumes of Percoll in the original procedure.

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 by means 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 observe any substantial differences between yields of separations, when comparing Percoll and Percoll PLUS. However, gradual failing of separations on Percoll appeared after about three months of its usage, whereas Percoll PLUS still worked after one year.

Combination of modified FPP and Percoll PLUS

Two-step separation resulted in the yield of about 20-60 % cells compared to one-step Percoll fractionation (i.e. $5\text{-}25 \times 10^4$ cells per single rat lung), but substantially diminished well-observable cell adherences to sides of employed probes. These adherences were located in density boundary lines between density Percoll layers resembling to beer rings on glass. 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.

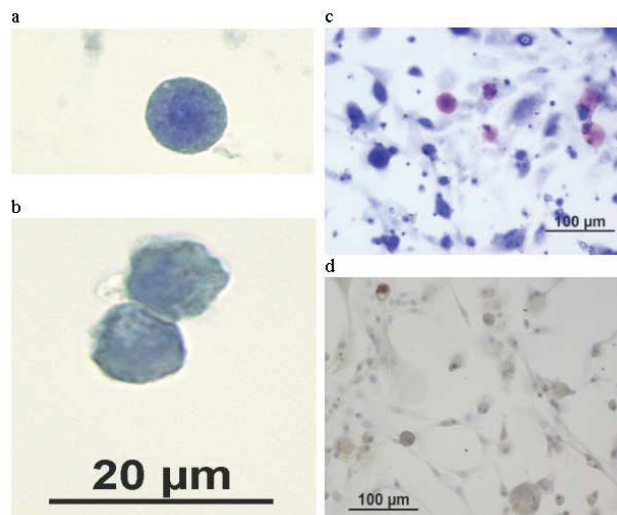


Fig. 1. Morphology of cultivated lung mast cells. a, b, c – six-day cultivation, d – three-day cultivation; a, b – comparable toluidine blue staining after hypoxic or normoxic cultivations, respectively; c, d – cells after one-step separation using Percoll PLUS only; c – purple metachromatic mature and blue immature 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”).

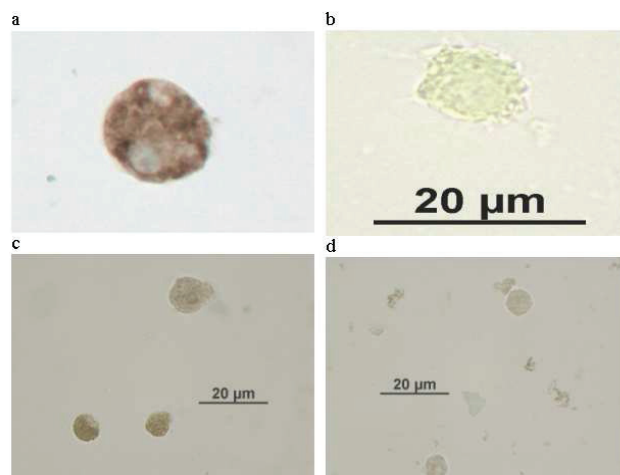


Fig. 2. Immunohistochemical detection of c-kit with LMC. a,c – c-kit-positive cells; b,d – negative controls; a,b – six-day cultivation; c,d – three-day cultivation.

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.*, *Staphylococcus epidermidis* (necessity of gloves usage during mast cell preparation). Nevertheless, frequent contamination can sometimes occur when adding pig pancreatic elastase I (lung cell extracting enzyme) without mixing to the PI medium (crystal of enzymes remain in bottom of stock solution).

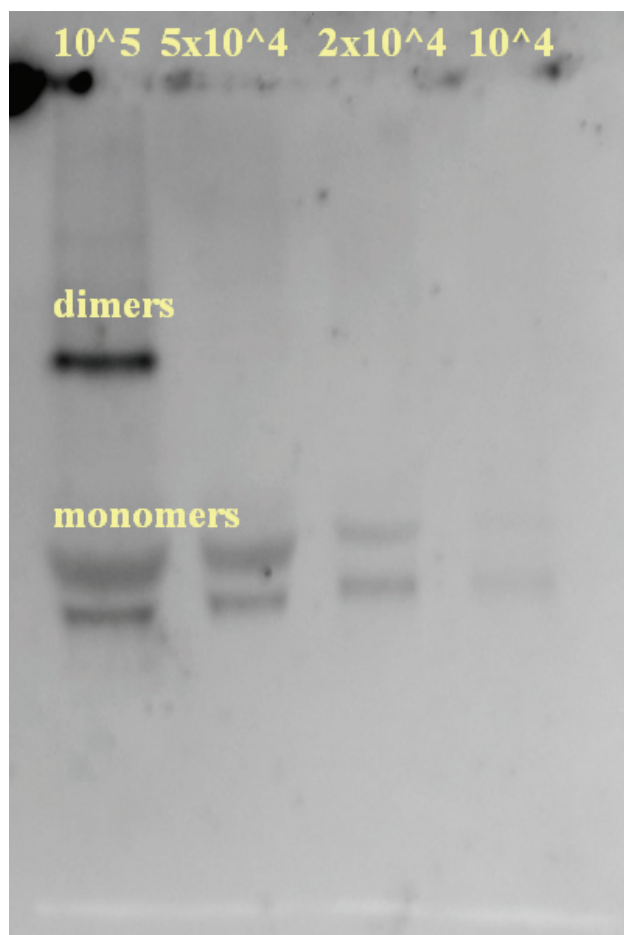


Fig. 3. Blotting detection of MMP13 in exosomes from model cell line RBL-2H3. MMP13 was still detected in minimum amount corresponding to 10^4 of source RBL-2H3 cells. We can also observe single band with MMP13 dimers (caused by low dilution of preparation with sample buffer), and double-bands containing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

Morphology of separated rat LMC cultivated after separation

In accordance with findings of Wu *et al.* (2008) a majority of LMC separated by our procedures exhibited immature morphology (Figs 1 and 2). Some preparations of LMC included among others also the cells, which seemed to contain two or several nuclei. This apparent nuclear duplicities or multiplicities were possibly caused by nuclear segmentation (cf. Discussion).

Surprisingly, some fibroblast-like cells were c-kit positive (Fig. 2) indicating thus morphological but not phenotypic deviations. However, these cells and fibroblast impurities grew more rapidly than spherical cells. Fibroblast-like cells were also observed after some one-step Percoll separations (Figs 2b and 2d) and during model allogenic reaction (Table 2). Proliferation of spherical cells in LMC tissue culture usually ceased

Table 2. Model cultivation of separated cells.

	Experimental values and calculated limits ^a			Cell proliferation and morphology		
	n3*	n6*	S.D.	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

^aSignificant 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; **S.D.** – group-related standard deviations. For additional comments see last section of Results.

^bdiv/day – Proliferation rate (**PR**) denoting number of cell divisions per day ($PR = \log(n6*/n3*)/(\text{days} \times \log 2)$, where days = 6-3 = 3). ^ccell 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 (original size of freshly isolated LMC); 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. ^drows including data: 1-4 – cultivation with SCF, IL-3, IL-6; rows 5-8 – in addition to the preceding cytokines we added filtered (cut off limit 0.2 μm) 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.

after 10-15 days of cultivations, whereas the fibroblast and fibroblast-like cells still proliferated.

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 (Fig. 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 (Fig. 3 and Discussion).

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 was preceded by three-day relaxation period (mean cell numbers n_6^* with calculated limit n_3^+ in Table 2). The required optimal size and shape was mostly found in the cases of proliferation rate lower than 0.35 divisions per day. 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 with “antibiotic antimycotic solution” and ii) significantly lower cell growth ($p < 0.05$, paired t-test was 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, and 8) achieved significantly higher proliferation rate ($p < 0.05$) and more deviated morphology (possibly due to allogenic reaction of residual lymphocytes) than in the culture with autologous 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 10 % 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 10 ml of medium) to the cultivation medium with samples 5-8. This addition of medium derived from RBL-2H3 cells 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 (rows 3 and 7 in Table 2).

Discussion

Comparison of our LMC separation with related methods

Since the separation of rat LMC is poorly described in the 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-term cultivations

(Willheim *et al.* 1995). Hence, the disinfection of elutriation rotor is still carried out simply by a 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 an interesting supplement to our future method improvement. However, the immediate improvement is complicated by a small 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 result 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 (cf. Methods), ii) effect of antibiotics and iii) antifungal and bactericidal effects of proteolysis.

Our introductory microbiological and microscopical screening concerned samples of mast cells 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 vancomycin. However, both these antibiotics triggered histamine release in some cases (Nessi *et al.* 1976, Chiang *et al.* 1992, Nabe *et al.* 1999, Toyoguchi *et al.* 2000) and thus they do not fit to our experiments with mast cells. Subsequently, we decided to introduce ampicillin to the 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.

Pig pancreatic elastase 1 (PPE1) is used together with other enzymes in the extraction of LMC. Substantial part of PPE1 is present in the bottom of its stock bottle in a form of crystals. Consequently, mixing of liquid in this stock bottle enables complete aliquot transfer of this enzyme to extraction mixture, where crystals are dissolved. Since we observed the effect of such bottle mixing on sterility of LMC tissue culture in some our experiments (cf. Results), we proposed bactericide effect of PPE1. However, we did not find any evidence of such bactericide effect of PPE1 in the literature. Only bactericide effect of other elastase, i.e. human neutrophil elastase, was confirmed (HNE; Miyasaki *et al.* 1991, Newman *et al.*

2000). In accordance with our BLASTP search for HNE sequence similarities, PPE1 contained the second most similar segment among vertebrate pancreatic elastases, whereas the segment of pancreatic elastase sequence of *Xenopus laevis* origin, but not human one, was the most similar (the search was performed in time before the paper submission). This fact suggested marked convergent, if not even functionally driven phylogenetic changes in PPE1 sequence. It appeared to be important in spite of marked sequence difference between PPE1 and HNE-related neutrophil elastase family following from our PSI BLAST/Clustal W-derived phylogram (data not shown). Consequently, the found superior sequence similarities between PPE1 and HNE supported our opinion that PPE1 stock solution mixing represent a one of the important steps necessary for sterility of LMC tissue culture.

Proliferation and morphology of LMC

In accordance with literature the majority of our LMC represent immature cells (Wu *et al.* 2008) enabling usually broader variation 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 (originating from different Wistar rats) generating cytokines. In accordance with expression studies of model human mixed lymphocyte 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 (Fig. 2) is related to morphology of more rapidly growing rat basophilic leukemia cell line RBL-2H3, representing widely used model of mast cells. Nevertheless, the addition of 10 % medium from six-day cultivation of RBL-2H3 to medium with LMC 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 cells (Suzuki *et al.* 1998, Onose *et al.* 2008) participate in such modulation or if we can alternatively assume the effects of membrane particles of 60-90 nm diameter called exosomes released by both mast cells and model RBL-2H3 cells (Skokos *et al.* 2002,

Laulagnier *et al.* 2005).

LMC looking like double- or multi-nuclear cells were observed in some samples (Fig. 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 does not change the density of large (toluidine blue-positive) histamine granules with a diameter of 800-1000 nm seems 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 the presence of MMP13 and perhaps other secreted molecules induced by hypoxia in membrane compartment distinct from large granules (see Results and Fig. 3). Based on presented data this compartment is composed of small vesicles with diameter of about 40-80 nm (Theoharides *et al.* 2007), representing precursors of the separated exosomes (Skokos *et al.* 2002). The investigated molecule of 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 initiating thus collagenolysis (Weingarten and Feder 1986, Novotná and Herget 2002). Increased collagenolysis in peripheral pulmonary arteries is then probably one of the important mechanisms that trigger pulmonary vascular remodeling in chronic hypoxia (Vajner *et al.* 2006).

Conclusions

We proposed here a new modification concerning Percoll PLUS solutions, which is based on initial "asymptotically isotonic" 100 % Percoll solutions (see Results). This modification enables us to prepare any well-separating Percoll PLUS gradients without measuring of osmotic pressure, which usually causes problems with Percoll PLUS losses or keeping of sterility of this separation medium. Similarly, whole our LMC separation is suitable for sterile cultivations, which last at least six days. In comparison with the LMC separation based on magnetic beads, our separation is less effective and selective, but on the other hand does not cause the artificial LMC activation by separating antibodies (see Discussion). Morphological analysis of cultivated LMC using light microscope suggests extended changes when mixing allogenic fractions of separated LMC, but less

visible changes in the case of hypoxia. In accordance with our study of model RBL-2H3 exosomes, we assume that the reason for less visible changes after hypoxia would not insist in absence of any LMC response, but more likely in the amplification of membrane vesicles observable only by electron microscopy.

Conflict of Interest

There is no conflict of interest.

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Abbreviations

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

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