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Investigation on the possibilities for *ex vivo*- and *in vitro*-derivation of initial myeloid and lymphoid precursors from hematopoietic and non-hematopoietic stem/progenitor cells

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Initial myeloid and lymphoid differentiation of hematopoietic and non-hematopoietic stem/progenitor cells by appropriate ex vivo- and in vitro-incubation was evaluated. So, hematopoietic stem/progenitor cells from mouse spleen were ex vivo-incubated in the presence of different interleukins, as well as of various combinations of them. Non-hematopoietic mouse embryonic stem cells (mESCs) were in vitro-incubated in the presence of GM-CSF and malignant antigens of human cervical carcinoma cells HeLa. Analogically, mouse embryonic fibroblasts from line 3T3 were pre-incubated in the presence of malignant antigens from mouse myeloma cells. Possibilities for derivation of myeloid and lymphoid cells by hematopoietic and non-hematopoietic cellular progenitors were proposed. The literature ability for production of immune molecules, including membrane glycoprotein receptors and antibodies/immunoglobulins by initial myeloid and lymphoid cells, derived from hematopoietic, as well as by non-hematopoietic cellular progenitors was accepted when appropriate factors are available. Because the produced antibodies are out of the germinative centers of the specialized lymphoid tissues and organs, control of their function is very important, for escape of chronic inflammatory processes. Realistically, this control proved the role of small ions and molecules, by direct and/or indirect influence on different intra- and extra-cellular inter-molecular interactions in various cascade regulatory pathways.

Key words: Hematopoietic and non-hematopoietic stem/progenitor cells, cellular differentiation, immune molecules, incubation conditions.

INTRODUCTION

In immune response, expansion of effector lymphocytes is a critical process for effective defense against pathogens and neoplasm. Viral infection and malignant transformation of cells can induce the expression of the MHC class I chain-related (MIC) molecules – MIC-A and MIC-B, as well as the UL16-binding proteins (Mistry and

O'Callaghan, 2007). These molecules are recognized by the NK cell activating receptor NKG2D. Ligand binding by this receptor can signal target cell killing (Lanier, 1998; Moretta et al., 2002; Waldhauer and Steinle, 2008). Efficient NK cell activation and function in response to viral infection or tumor cells is critically dependent on the NKG2D pathway and may help design effective strategies to improve the outcome of cancer therapy (Zhu et al., 2010). Cytokines IL-2, IL-7, IL-9, IL-15 and IL-21 bind to the common cytokine receptor γ-chain. Such interactions lead to NK cell development and proliferation as these cytokines have a role in the JAK/STAT pathway (Bianchi et al., 2000; DiSanto et al., 1995; Xiong et al., 2008). By contrast, IL-18 transduces signals into NK cells through its own receptors, that do not use a common γ-chain receptor, and can activate NF-kB through myeloid differentiation primary response gene 88 (MyD88)-mediated pathway (Adachi et al., 1998). While IL-18 was discovered in the immune system, it is ubiquitously expressed not only in hematopoietic cells, but also in non-hematopoietic cells, including cardiac myocytes, keratinocytes, intestinal epithelial cells, retinal cells and many endocrine cells, although the biological roles of IL-18 in the non-hematopoietic cells remain unclarified. This suggests that IL-18 is not merely a cytokine inducer and plays important biological roles, for example, in metabolic homeostasis of various cells. Especially, discovery of inflammasomes suggested that IL-18 has a role as a responder molecule against stresses caused by various danger signals. Exogenous IL-12 has been found to markedly accelerate appearance of cells with phenotypes of NK cells. These lymphocytes are one of the cells participating in the first line of defenses against pathogens and altered tissues such as neoplasm. NK cells exert rapid cytolytic activity upon sensing the first signals caused by infection or transformation, and the naturally equipped cytotoxic activity of NK cells may be important for presentation of cellular debris or degraded pathogens to dendritic cells (DCs), which further process them and present antigens to T-lymphocytes. On the other hand, a rapid lymphoid-restricted (T, B- and NK) reconstitution capacity *in vivo* and completely lacked myeloid differentiation potential of both *in vivo* and/or *in vitro* has been reported in stem cells from bone marrow material of adult laboratory mice (Kobari et al., 2000).

In order to accomplish their roles, NK cells may not be necessary to expand by proliferation (Lanier, 1998; Moretta et al., 2002). However, it was demonstrated that there is a subset of highly proliferative NK cells. The last have shown properties of DCs and used to be called as IFN-g-producing killer DCs (IKDCs) or NK-DCs (Waldhauer and Steinle, 2008; Zhu et al., 2010). There have been debates for many years about phenotypic and functional identification of these NK cells, and now they are designated as pre-mature NK cells (pre-mNKs), which are considered to be intermediate cells developing to mature NK cells (mNKs cells). The origin of mNK cells is from hematopoietic stem cells, and their phenotype has been characterized as $DX5^{+}NK\text{-}1.1^{high}B220^{low}CD11c^{+}CD122^{+}$. On the other hand, the origin of pre-NK cells is rather complicated, and *in vitro*-activated mNK cells exhibit similar characteristics of pre-mNK cells with phenotypes of $DX5^{+}NK\text{-}1.1^{high}B220^{high}CD11c^{+}CD122^{+}$, but the discrepancy in mechanism of generation of pre-mNK cells in *in vitro*- and *in vivo*–conditions remains to be overcome (Adachi et al., 1998; Kobari et al., 2000). Regardless, the pre-mNK cells are considered to be the precursors of mNK cells. Hence, resting naïve NK cells, highly proliferating NK cells with pre-mNK phenotypes and non-proliferating NK cells with phenotypes of $NK\text{-}1.1^{low}$ $B220^{high}$ $Ly6C^{low}$ $Sca\text{-}1^{very\ high}$, exerted definitely different phenotypes and functions.

In this connection, the main goal of the current study was directed to derivation of initial myeloid and lymphoid precursors from hematopoietic and non-hematopoietic types of stem cells, by application of appropriate incubation techniques.

MATERIALS AND METHODS

Isolation, purification and cultivation of cells from mouse spleen

NK cells were isolated from previously prepared suspension of spleen from experimental Balb/c mice. For derivation of NK cells, the received suspension was treated with buffer for erythrocytes lysis, and subsequently - CD4, CD8, CD19 MACS microbeads, which are non-toxic bio-degradable nano-particles for detection and separation of cellular sub-populations. NK markers were proved by application of flow cytometry assay. The obtained population of NK cells was then *in vitro*-incubated and stimulated with different interleukins or various combinations of them. The cells were picked-up and analyzed at day 4 post incubation (p. i.).

All experiments were conducted in accordance with the ethical laws, and were approved by the Animal Research Committee of Hyogo College of Medicine. Animals had free access to food and water, and were kept in pathogen free environment with a 12 h/12 h light/dark cycle. A constant temperature of 25°C was maintained.

Recombinant mouse IL-18, IL-15 and IL-18 were all commercially obtained by GlaxoSmithKline PLC (Research Triangle Park, NC), PEPROTECH Rocky Hill and NJ R&D, Minneapolis MN 55413, USA, respectively.

The resulting isolated cells were incubated for 4 days in RPMI1640 medium (Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% FBS (BioWest, Nuaillé, France), L-glutamine, 100 units/mL penicillin, 100 mg/mL at 37°C in a humidified atmosphere with 5% CO_2. NK cells were divided into two

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groups. The cells in the first group were cultured in presence of 10 ng/mL IL-15 and/or 100 ng/mL IL-18. The cells in the second group (control group) were cultured in RPMI1640 medium with thesame supplements but with no presence of IL-15 and/or IL-18. Before analyzing the cell proliferation or expression of the markers, the cells were harvested and their viability was monitored using Trypan Blue dye exclusion test.

Microscopy techniques for determination the type of cellular clusters

For observation, the processes of cellular proliferation, type of clusters formation or their lack, light microscope Olympus CK2 was used. The photos were prepared by reflection photo-apparatus Canon (EOS Rebel T5i DSLR Camera with 18-55 mm IS STM Lens Kit).

Derivation of initial myeloid and lymphoid cellular progenitors by appropriate in vitro-incubation of mESCs and mouse embryonic fibroblasts

Normal mouse embryonic stem cells (mESCs) were incubated in the presence of GM-CSF and malignant antigens, received by addition of cultural fluid from previously incubated human malignant cervical carcinoma cells HeLa, after subsequent centrifugation and filtration. Analogically, normal embryonic fibroblasts from line 3T3, derived from Balb/c mice, were pre-incubated with cultural fluid from previously incubated mouse malignant myeloma cells. All cells were incubated at 37°C in incubator with 5% CO_2 and 95% air humidification, in Dulbecco's modified minimal essential medium (DMEM) (Sigma-Aldrich), supplemented with 10% fetal calf serum (FCS) (Sigma-Aldrich), 100 IU/ml penicillin (Sigma-Aldrich) and 100 µg/ml streptomycin (Sigma-Aldrich). Fixed light microscopy slides were prepared by treatment with Ethanol (Sigma-Aldrich), subsequent washing with PBS (Sigma-Aldrich) and stained by Giemsa-dye (Sigma-Aldrich).

ELISA protocol

Cells of the three culture types (normal mouse embryonic fibroblasts 3T3; mouse malignant myeloma cells, as well as a mixture of both cellular types) were lysed by treatment of previously-prepared cell suspensions with 10% tri-chloroacetic acid (Cl_3CCOOH) and 0.48 M solution of K_3PO_4. All samples were subjected to ELISA. For this goal, slight modifications of the method of Mitzutamari et al. (1994) were made. In general, 1000 ng ganglioside dry substance (Sigma) was diluted in 100-ml methanol. Aliquot of 100 µl from this solution was pipetted into 96-plate wells, containing the tested biological material. Subsequently, the wells were air-dried and blocked with BSA-PBS (Sigma) (1% bovine serum albumin in phosphate-buffered saline) for 1 h. After six-fold washing with PBS, 100 µl of each one of the prepared lysates from the cell cultures, described above, diluted 1:20 to 1:5000 in PBS were added to each well and incubated overnight. Subsequently, the plates were washed six-fold with PBS. Binding was detected by following 2 h incubation period with BSA-PBS (Sigma) diluted (1:3200) peroxidase-conjugated goat anti-human IgG antibodies (Bul Bio Ltd., NCIPD, Sofia). All incubations were performed at 4°C. Thereafter, the plates were washed six times with PBS. Color development was achieved in a substrate solution, previously prepared from 15 mM O-Phenylenediamine and 0.015% H_2O_2 in 0.1 M sodium acetate buffer (0.2 M CH_3COONa/0.2 M CH_3COOH; pH 5.0) at 20°C. For determination of the titers of gangliosides, instead of ganglioside solution, serum, previously proved to contain specific anti-GM3 antibodies, was added to the tested samples. The reaction was stopped after 30 min with 50 ml of 1N H_2SO_4 and the optical density (OD) was assessed spectrometrically at 490 nm on ELISA-reader (TECAN TM, Sunrise, Austria). Non-specific bindings (OD value in a well not containing the specific molecule in the respective probe) were subtracted for each measurement. The data were considered strongly positive, when the mean OD exceeded 2 ± SD (standard deviation), compared with the controls. The standard error of mean varied between ± 0.01 and ± 0.1. For the best reliability, the procedure was repeated three times.

RESULTS

Different stages of differentiation of hematopoietic stem cells to NK cells have been noted, depending on the time and factors of differentiation/cytokines (Figure 1). A proof about differentiation in NK cells is the noted formation of cellular clusters. Formation of clusters of highly proliferative NK cells in the presence of IL-15/IL-18 (Figure 1b), as well as in the presence of IL-15/IL-18 + IL-12 (Figure 1c), could be explained by the expression of various molecules related to the effector functions, compared to the control non-treated cells (Figure 1a).

Analogically, initial differentiation to lymphoid and myeloid lines was noted in pre-incubation of mESCs in the presence of GM-CSF plus malignant antigens from malignant human cervical carcinoma cells from line HeLa (Figure 2b), compared with the control non-treated culture of them (Figure 2a). Similar changes were noted in pre-incubation of mouse embryonic fibroblasts from 3T3 cell line in the presence of malignant antigens of mouse myeloma cells (Figure 2d), compared with the non-treated control culture of the same cells (Figure 2c). The presented results proposed immunogenic properties of non-myeloid and non-lymphoid cellular types in appropriate conditions such as the presence of malignant and/or viral antigens along with appropriate immunomodulators. Higher cluster-formation properties of ESCs could be noted (Figure 2b), compared to the embryonic fibroblasts, where initial myeloid-like cellular precursors are prevailing (Figure 2d).

At all dilutions of the tested samples, an increase in the titers of both gangliosides and anti-ganglioside antibodies could be noted (Figure 3). At the lowest dilution (1:40), the highest titers of both gangliosides and anti-ganglioside antibodies was observed in the sample of malignant cellular culture, compared to the lysates of normal cells and of the mixed culture. At the higher dilutions of the same samples, an increase in the titers of gangliosides, as well as in the anti-ganglioside antibodies was noted. At all other dilutions (1:100; 1:200 and 1:400), the titers of gangliosides in the extract of malignant cellular culture was lower compared to the other culture lysates. At the highest dilution of 1:400, the highest titer was detected in the extract of normal cell culture (Figure 3b). Analogical tendency was seen for the titers of anti-ganglioside antibodies with increasing of the lysate dilutions; however, here in the highest dilutions of 1:200 and 1:400, the highest titers were observed in the samples from the mixed culture extract, but only in dilution 1:100 was noted

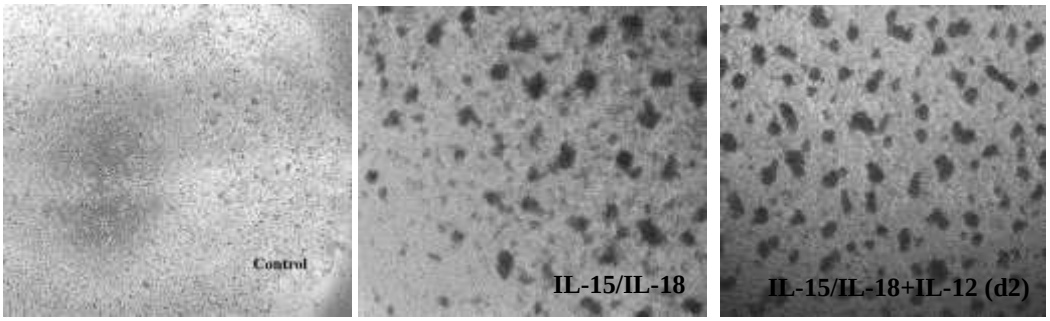

Figure 1. Formation of clusters of cells between stimulated by different combinations of interleukins (IL-15, IL-18, IL-12) NK cells at day 4 p. i., derived from hematopoietic stem/progenitor cells of mouse spleen: a – control non-treated hematopoietic stem cells; b - treated with IL-15 and IL-18; c – treated with IL-15 and 18 plus IL-12, magnification: 40 x.

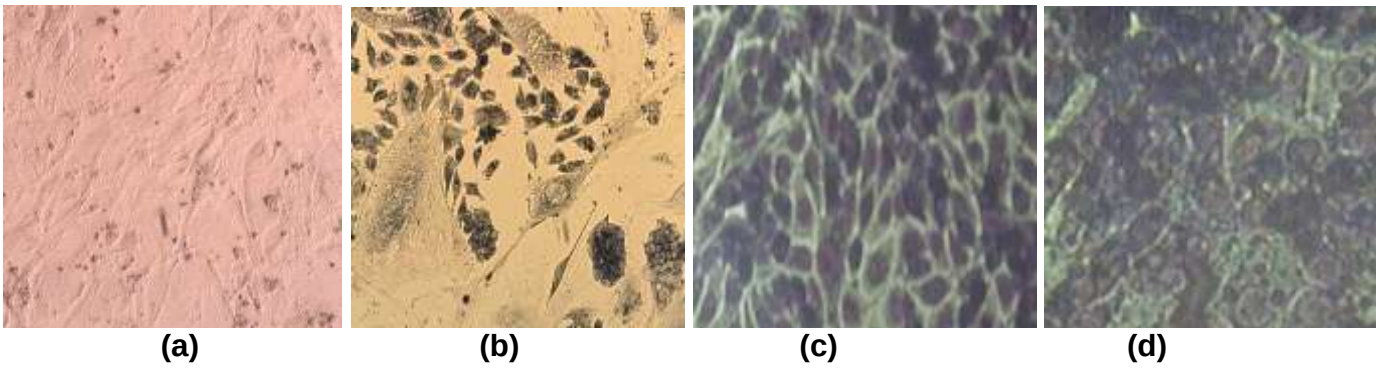

Figure 2. *In vitro*-derived initial myeloid-like and lymphoid-like progenitors: a – control non-treated culture of mESCs (native preparation, magnification: x100); b - culture of mESCs, pre-incubated in the presence of GM-CSF plus cultural fluid from previously incubated in it human malignant cervical carcinoma cells HeLa (Giemsa-dye, magnification: 100 x); c – control non-treated culture of mouse embryonic fibroblasts from 3T3 cell line (Giemsa-dye, magnification: 200 x); d – culture of mouse embryonic fibroblasts from 3T3 cell line, pre-incubated in the presence of cultural fluid from previously incubated mouse malignant myeloma cells (Giemsa-dye, magnification: 200 x).

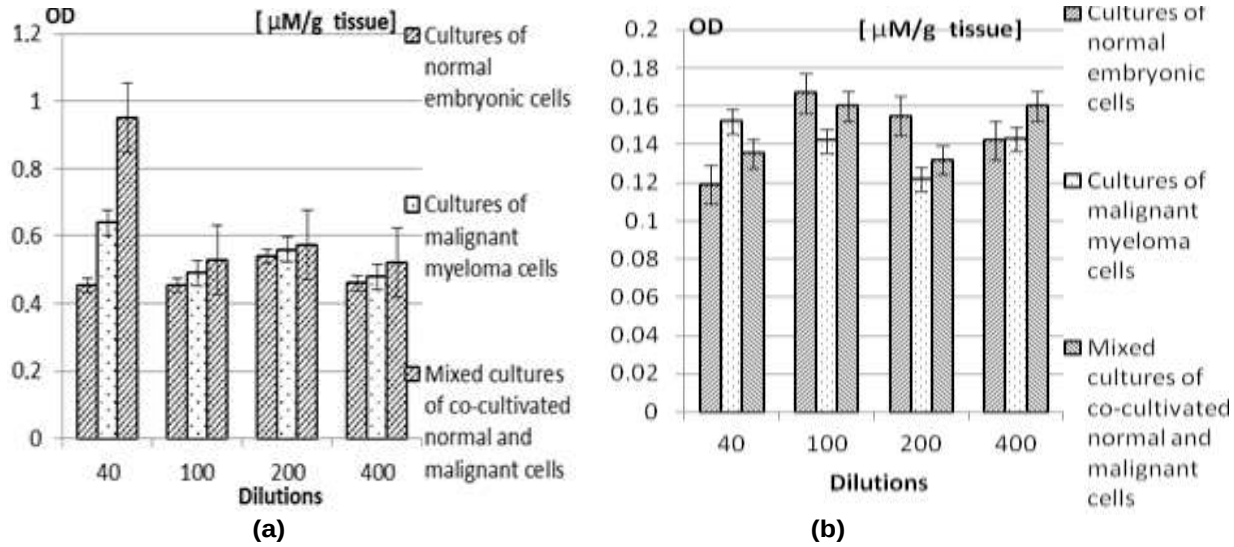

Figure 3. Average titers of anti-ganglioside antibodies (a) and gangliosides (b) in extracts from experimental *in vitro*-incubated cultures of normal mouse fibroblasts from embryonic cell line 3T3. mouse malignant myeloma cells, and mixed cultures of both cellular types. OD – optical density.

the highest titer in the extract of normal cell culture (Figure 3a). As a whole, however, in all cases the titers of anti-ganglioside antibodies were higher compared with those of the gangliosides. This tendency could be established at each dilution of the extract in each respective cell culture.

DISCUSSION

Studies on the stem cells' biology are often focused on their self-renewal and differentiation (Smith, 2001; Smith and Boulanger, 2002). The efficiency of DNA-repair varies among different stem cell types. Of key importance is the coordinated oncogenes and tumor-suppressor genes activity (Rubin, 1997; Vaziri and Benchimol, 1998; Vogelstein and Kinzler, 2004). Signalling strength, kinetics and specificity of these pathways is modulated at many levels by distinct regulatory proteins. Stimulation of NK cells by IL-18 alone could prime NK cells to up-regulated expression of various molecules, both in the cell surface and cytoplasm (Tamzalit et al., 2014). These cells have also been suggested to be able to recruit effector T-lymphocytes to the tumor site, because IL-18-primed helper NK cells have been shown to promote recruitment of $CD8^+$ T cells to the tumor microenvironment (Mitchell et al., 2005). In our study, the observed signs of initial myeloid-like and lymphoid-like cellular phenotype in co-cultivation with malignant cells are in support with the literature sources about the influence of appropriate factors on the differentiation (Okubo et al., 2000). These data once again supports the proven role of GM-CSF and *STAT*-genes for the normal myeloid and lymphoid cellular differentiation (Coffer et al., 2000). These results are in agreement with the indicated initial myeloid and lymphoid differentiation of non-transfected normal mESCs by activation of genes from *STAT*-family (Kyba et al., 2003; Wang and Bunting, 2013). One of the hypotheses was based on that proved in the scientific literature possibly about appearance of initial lymphoid and myeloid differentiation signs in sub-populations of immature stem-like cells. Other explanations were based on "intrinsic immunity" changes, as internal protection of the cell in response to its infection (Murray et al., 2018; Yan and Chen, 2012), as well as eventual appearance of early signs of immune cell differentiation of immature embryonic cells in the presence of appropriate immunomodulators (Kobari et al., 2000; Kyba et al., 2003; Wang and Bunting, 2013). In a similar way, the current data also suggest a possibility for production of antibodies/immunoglobulins by non-lymphoid types of cells in appropriate conditions, which is in agreement with the research on this topic (Bebbington, 1991; Cho et al., 1999; Keller, 2005; Nematpour et al., 2017; Popi et al., 2009). However, because these antibodies are probably out of the germinative centers of the specialized lymphoid tissues and organs, the control of their functions is very important for escape of chronic inflammatory processes, which could lead to appearance of malignancies or of degenerative changes. The key role of small ions and molecules in this control, such as the acidic glycospingolipids gangliosides, by direct participation and/or indirect influence on various intra- and extra-cellular inter-molecular interactions in different regulatory pathways has been proven (Natalizio et al., 2001).

Conclusion

Possibilities about derivation of initial myeloid and lymphoid precursors from hematopoietic and non-hematopoietic stem cells, as well as from early differentiated embryonic fibroblasts, were shown. The differentiation of both types of stem cells, along with early embryonic fibroblasts, could be achieved by appropriate *ex vivo*- and *in vitro*-incubation in the presence of respective conditions, depending of the concrete origin of cellular progenitors. In this connection, a possibility for production of membrane glycoprotein receptors and antibodies/immunoglobulins by non-myeloid and non-lymphoid cellular types was proposed, when malignant cells or antigens, viruses or viral antigens, as well as appropriate immunomodulators are available. However, because the resulting antibodies are out of the germinative centers of the specialized lymphoid tissues and organs, control of their function is very important, for escape of chronic inflammatory processes. The role of small ions and molecules as gangliosides has been proven as key in this control.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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