Human umbilical cord blood derived serum and platelet-rich plasma can replace fetal bovine serum to induce human monocytes into dendritic cells

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Abstract

Introduction: Dendritic cell therapy is a promising therapeutic therapy for cancer. Various methods have been developed to culture and expand dendritic cells \textit{in vitro}. However, most methods have used fetal bovine serum (FBS)-supplemented media to induce and expand monocytes; cells prepared by these methods cannot be used in the clinic. Therefore, this study aims to develop new methods to produce dendritic cells (DCs) from monocytes using serum-free medium. Methods: mononuclear cells (MNCs) were isolated from human umbilical cord blood by gradient centrifugation. They were then induced into DCs using 3 kinds of inducing media: M1 (medium supplemented with FBS), M2 (medium supplemented with human serum (HS) from umbilical cord blood), and M3 (medium supplemented with platelet-rich plasma (PRP) from umbilical cord blood) at 10% supplement. The MNCs were induced to immature DCs (iDCs) by granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4, and matured by tumor necrosis factor (TNF)-alpha. The phenotype of the DCs were evaluated by flow cytometry, immunohistochemistry, and \textit{in vitro} phagocytic assay. Results: The results showed that all cells in the groups exhibited the shape of dendritic cells.
Immunohistochemistry analysis showed that the mature cultured cells in the 3 kinds of media (i.e. supplemented with either FBS, HS, or PRP) were all CD86+HLA-DR+CD14−, representative of mature DC (mDC) phenotype. DCs cultured in HS and PRP media also exhibited FITC-Dextran phagocytosis and showed IL-12 gene expression similar to those DCs cultured in FBS medium. **Conclusion**: The results of the present study suggest that HS or PRP can replace FBS to produce DCs in vitro for clinical use.

**Keywords**

Human cells, Human cord blood, Human serum, Platelet-rich plasma, Platelets, Serum-free media

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**Introduction**

Dendritic cells (DCs) are specialized antigen-presenting cells of the immune system, presenting antigen to both B cells and T cells (Steinman, 1991). Myeloid dendritic cells were first detected by Ralph Steinman in 1973 in the spleen of mice (Steinman and Cohn, 1973). Dendritic cells are often differentiated in vitro from the spleen, bone marrow, peripheral blood mononuclear cells (MNCs), or MNCs from cord blood. In order to have large numbers of cells, the DCs are usually proliferated and differentiated in vitro from precursor cells. Stimulatory factors that are often used are granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 (Inaba et al., 1992; Young et al., 1995). DCs are stimulated by tumor necrosis factor (TNF)-alpha and cancer antigens. In principle, DCs will process and present foreign antigens on the major histocompatibility complex (MHC) class I and II.

The culture medium supplemented with fetal bovine serum (FBS) is commonly used in DC culture. Indeed, FBS in the culture medium is a large source of foreign proteins and may be presented on the membrane surface of DCs and cause undesirable immune responses (Haase et al., 2005). Moreover, culture media containing FBS exhibits a number of risk factors, such as prions, viruses and/or other immune responses that may occur (Haase et al., 2005; Lutz and Rossner, 2007; Toldbod et al., 2003). Furthermore, many of the factors present in the FBS composition affect the maturation of DCs. These unknown issues may be harmful for the clinical application of DCs to treat adenocarcinomas (Lutz and Rossner, 2007).

Numerous studies on the effect of media on the maturation of cell culture in vitro have been conducted. Many results have suggested that FBS can be replaced by human serum (HS) during culture of cells (Anton et al., 1998;
Many serum-free media have been studied for culturing DCs for use in clinical applications; these media include AIM-V, X-VIVO 15, and X-VIVO (Peng et al., 2005; Royer et al., 2006; Tkachenko et al., 2005). Most of these studies aimed to establish a type of culture media without FBS, but the effects on differentiation and maturation of DCs remain to be addressed.

The present study aimed to assess the effects of media containing autologous HS platelets, platelet-rich plasma (PRP), and FBS on the induction of MNCs to DCs.

**Materials-Methods**

**Human serum and platelet-rich plasma preparation**

All umbilical cord blood samples were negative for human immunodeficiency virus (HIV), hepatitis B virus (HBV), and hepatitis C virus (HCV) prior to enrollment, and were obtained from the donors with consent forms. Samples of cord blood were collected into 50-ml centrifuge tubes without anti-coagulation. Blood will coagulate in the lower layer of the tubes after settling at room temperature for 2 hours; the serum is the yellowish layer directly above. Serum was collected by centrifugation at 10,000 g for 10 minutes at 4°C. Subsequently, the serum was inactivated in a 56°C thermostat bath for 30 minutes, and stored at -80°C until use.

Using the same umbilical cord blood sample, 50 ml of blood was collected in a 50-ml centrifuge tube with anticoagulant. The blood and anticoagulant ratio was 6:1. Blood cells were used to isolate nuclear cells were used to generate PRP. In brief, the tube of blood was centrifuged at 300 g for 15 minutes. After centrifugation, the plasma was transferred to another 50-ml centrifuge tube to generate PRP, then centrifuged at 400 g for 15 minute at room temperature and 1/3 of the floating cell suspension was retained. Then, cells were re-suspended with the remaining plasma and centrifuged to obtain PRP. Next, 10% calcium chloride (CaCl₂) was added to the PRP suspension and incubated at 37°C for 30 minutes to remove the fibrin gel and release the platelet factors. Blood samples were diluted with PBS (1:1 ratio) and placed onto a Ficoll-Paque (GE Healthcare, Uppsala, Sweden) layer in a 50-mL centrifuge tube. The tube was centrifuged at 450 g for 45 minutes to separate the cell segments; mononuclear cells were present in the top Ficoll-Paque layer.

**Monocyte isolation and induction**

Monocytes were obtained from umbilical cord blood. All umbilical cord blood samples were negative for HIV, HBV, and HCV prior to enrollment, and obtained from the donors with consent. MNCs from cord blood were isolated by gradient
centrifugation on Ficoll-Paque (density: 1,077 g/ml; GE Healthcare). MNCs were washed with culture medium before they were plated in 6-well plates (5x10^6 cells/well) supplemented with 20 ng/ml human GM-CSF and 20 ng/ml human IL-4 (both from Santa Cruz Biotechnology, Dallas, TX, USA). After that, cells were induced in that cytokine condition for 10 days.

After 10 days of culture, the immature dendritic cells (iDCs) were incubated in fresh culture medium with 20 ng/ml human GM-CSF and 20 ng/ml human IL-4, and cultured in 6-well plates at 10^6 cells/well. The iDCs were induced with 20 ng/ml TNF-α for 72 h to become mature DCs (mDCs).

**Phenotyping by flow cytometry**

The surface expression of CD14, CD86, and HLA-DR were evaluated for iDCs and mDCs by flow cytometry. In brief, 10^6 cells were separately stained with CD14-FITC, CD86-FITC, and HLA-DR-FITC antibodies at room temperature for 30 min in the dark. The cells were then wash twice to remove the extra antibodies. Finally, the stained cells were analyzed in a flow cytometer (FACSCalibur, BD Biosciences, San Jose, CA, USA) using the CellQuest Pro software (BD Biosciences) with isotype controls.

**Phagocytosis assay**

To evaluate phagocytosis of DCs, 5x10^4 cells were incubated for 24 hours with FITC-Dextran (1 mg/ml, Sigma-Aldrich, St Louis, MO, USA) in 100 μl culture medium at 37°C. Similar samples induced at 4°C were used as controls. After that, the cells were washed twice with cold PBS before flow cytometry analysis. Phagocytosis activity of DCs was evaluated as a population of FITC-expressed cells in the FACSCalibur flow cytometer using the CellQuest Pro software.

**Evaluation of IL-12 expression**

In brief, mRNA from mDCs was extracted using EasyBlue kit (iNtRON Biotechnology, Korea). DCs were analyzed for IL-12 gene expression by one-step reverse transcriptase (RT)-PCR Premix Kit (iNtRON Biotechnology, Korea). The primer sequences used were the following: IL-12 (305 bp), Forward primer: 5' G C T G G G A G T A C C C T G A C A C - 3', Reverse primer: 5' TTGGGTCTATTCGGTGTGT-3'; Beta-actin (304 bp), Forward primer: 5' A C A G T T T G T G G T G T A G G T-3', Reverse primer: 5' GAGGCGTACAGGGATAGCAC -3'.

**Results**

**DCs cultured in all 3 types of media exhibited similar shape**

DCs cultured in the three types of medium (i.e. supplemented with FBS, HS or PRP) all exhibited the same shape (**Fig. 1**). After 2 hours of culture, almost all the
cells adhered to the plate surface (Fig. 1A). We replaced the media to remove the non-adherent cells. After 7 days of exposure to GM-CSF and IL-4, the cultured cells in FBS, HS or PRP mediums were all elongated in shape and showed numerous small branching (dendrites) (Fig. 1B, C, D).

![Figure 1](image1.png)

**Figure 1.** The change of human umbilical cord blood MNCs into DCs after culturing in various types of media. (A) MNCs attach to the plate surface after 2 hours. (B) Dendritic cell shape observed after culture in FBS-supplemented medium at day 7; (C) Dendritic cell shape after culture in HS-supplemented medium at day 7; (D) Dendritic cell shape after culture in PRP-supplemented medium at day 7.

After 10 days, the cells appeared to have larger nuclei and longer tassels, as they clung to the culture surface. When transferred into an environment with TNF-α supplementation, some cells contracted and there were many small tufts. They are weakly attached and separated from the culture surface. In terms of shape, the DCs in the three types of media were not significantly different (Fig. 1 B, C, D).
The expression of DC marker was not significantly different among the 3 kinds of media

The mDCs in the experimental groups were evaluated for expression of CD14, CD86 and HLA-DR using CD14-FITC, CD86-FITC and HLA-DR-FITC antibody markers (Fig. 2, 3). Each marker was evaluated in triplicates and over 3 independent assays. Isotype antibodies were used as negative control samples.

The results from the phenotypic analysis of the DCs after culture in the 3 different media showed that for mDCs, the cells showed weak expression of CD14, while simultaneously strong expression of CD86 and HLA-DR. DCs in PRP-supplemented medium had the lowest CD14 expression (%) compared to the HS-supplemented and FBS-supplemented groups. However, the level of CD14 expression was not significantly different among the 3 culture conditions by statistical analysis (p>0.05, n=3). Similarly, HLA-DR expression in the groups were high and there was no statistically significant difference. The differences between batches occurred at the level of expression of CD86; propagated cells cultured in FBS-supplemented medium exhibited higher CD86 expression than the other 2 groups.
Figure 3. Immune phenotype of mDCs in media containing FBS, HS, or PRP. Top row: unstained cell samples; middle row: CD14-FITC staining (cell samples stained with CD14-FITC antibody); third row: CD86-FITC staining (cell samples stained with CD86-FITC antibody); forth row: HLA-DR-FITC staining (cell samples stained with HLA-DR-FITC antibody).
Phagocytosis of the DCs

DCs were cultured in a media and incubated with FITC-Dextran for 24 h (Fig. 4A). Phagocytosis was evaluated by measuring the dextran-FITC absorption of the cell. After induction, iDCs in different plots had a phagocytic capacity of 74.8±12.8%; 84.4±7.4% and 84.0±7.2%, respectively for the FBS, HS and PRP groups. Mature phagocytes, in turn, had a phagocytic capacity of 43.7±7.4%; 55.4±2.9% and 45.0±5.7%, respectively for the FBS, HS and PRP groups. Meanwhile, non-induced GM-CSF-, IL-4- and TNF-α- treated cells were very weak at phagocytosis.

Furthermore, the cells incubated at 4°C did not have any phagocytic capacity. These results suggest that induced-cells can perform the function of DCs. These cells show diminished phagocytic function as they mature, a feature of DCs in vivo. In the HS-treated and PRP-treated conditions, iDCs were significantly more phagocytic than those in the FBS-treated conditions. However, when induced with TNF-α, the phagocytosis ability of DCs in all conditions was significantly reduced. For statistical analysis, the phagocytosis abilities of iDCs and mDCs in the FBS, HS and PRP conditions were not significantly different (p=0.078; n=4).

Expression of IL-12 gene

IL-12 is one of the most important genes in mDCs for the production of T-cell-mediated cytokines. Typically, antigen-induced cells will express the IL-12 gene. For all three culture conditions, IL-12 expression was assessed by RT-PCR. The results showed that FBS, HS, and PRP samples showed a band with size of 305 bp (corresponding to the molecular weight of IL-12). This suggests that mDCs are capable of expressing the IL-12 gene. This result is similar to that of other authors (Canque et al., 2000; Kaliński et al., 1997; Ratta et al., 1998).

Figure 4. Diagram showing phagocytosis and IL-12 gene expression of DCs. (A) FITC-Dextran phagocytosis of immature and mDCs. (B) IL-12 gene expression of DCs; M (100 bp ladder); β-actin (304 bp); IL-12 (305 bp).
Discussion

Some studies have shown that the use of DCs induced by in vivo antigens may lead to the activation of immune cells and sometimes abnormal behavior; instead of presenting the target antigens, DCs may present other antigens. The presence of these other antigens may cause autoimmune reactions and disease. Numerous studies have reported that this non-specific immune response is associated with the use of FBS or fetal calf serum (FCS) in culture (Haase et al., 2005; Kadri et al., 2007; Lutz and Rossner, 2007). However, some pre-clinical studies have accepted the use of FBS supplementation in DC culture media. However, for clinical applications, the heterogeneous elements in FBS need to be eliminated as much as possible. Many published studies have succeeded in creating DCs with serum-free media but there have also been limitations (Napoletano et al., 2007).

In this study, we used culture media supplemented with HS and PRP from human umbilical cord blood instead of FBS. Our results suggest that MNCs derived from human cord blood can be differentiated into DCs when induced by differentiation factors (GM-CSF, IL-4, TNF-α, etc.) in media supplemented with HS or PRP, similar to that extent seen in FBS-supplemented conditions. Indeed, in our study, we first derived MNCs from the same umbilical cord blood and cultured them in media supplemented with the 3 different serum components. The cells adhered to the culture plates. According to our observation, the cells cultured in PRP-supplemented media attached more strongly than the cells in the other conditions. This may indicate that there are many factors in PRP that promote cell adhesion (Boyapati and Wang, 2006; Eppley et al., 2004; Tozum and Demiralp, 2003).

After being induced into mDCs in two stages, using GM-CSF, IL-4 and TNF-α, the cells exhibited DC-like shape (branches or dendrites). The characteristics of the DCs were similar to those of other types of DCs derived from different sources, such as bone marrow MNCs, peripheral blood, or umbilical cord blood (Bai et al., 2002). Cell differentiation typically progresses through the following stages: (1) formation of small clusters on the culture surface; (2) stretching and formation of small tassels; and (3) shrinking and detaching from the culture surface. These stages are similar to DC generation in vivo: (1) precursor DCs; (2) iDCs; (3) DC migration; and (4) DC maturation (Li et al., 2012).

Next, we evaluated the expression of specialized markers on mDCs. DC markers, such as HLA-DR, CD80 and CD86, play important roles in the function of DCs. In the body, DCs act as the initiator of immune responses. They phagocytose exogenous antigens and process them as small peptide fragments for binding to surface MHC-II molecules to present to T-cells (Palucka and Banchereau, 2012). The CD80/CD86 surface molecules act as costimulatory molecules which help DCs activate T cells via the CD28 receptor (Kapsenberg, 2003). Cells cultured in HS-supplemented or PRP-supplemented
medium, induced with GM-CSF, IL-4 and TNF-α, exhibited the phenotype and function of mDCs, similar to those cultured in FBS-supplemented medium. These cells show strong CD86 and HLA-DR expression but weak CD14 expression.

However, in the media supplemented with 10% PRP, the proportion of mDCs expressing CD86 was weaker than that of the other two groups. The cause of this may be that in the PRP-supplemented media, there are inhibitors of DC maturation. Numerous studies have shown that in addition to factors promoting the maturation of DCs (such as IL-10, CD154, heat-shock protein Gp96) (Czapiga et al.; Hagihara et al., 2004; Hilf et al., 2002), PRP contains factors that inhibit DC maturation (such as VEGF) (Oyama et al., 1998).

In phagocytosis testing, DCs in all conditions demonstrated functional phagocytic ability. Indeed, phagocytosis is one of the most important characteristics of DCs. In the immature stage, DCs recognize, phagocytose, and process the extrinsic antigens for presentation to immune cells (Savina and Amigorena, 2007). In the present study, iDCs showed strong ability of FITC-Dextran phagocytosis. After maturation, the DCs showed weaker phagocytic capacity. This result is similar to other studies (Lyakh et al., 2000; Romani et al., 1994; Steinman, 1991).

Another feature of DCs is the ability to secrete IL-12 cytokine for T-cell activation. In this study, the IL-12 gene expression level in mature DCs cultured under the different media regimens were evaluated. The results showed that in the HS- or PRP-supplemented conditions, DCs expressed IL-12 gene, as was the case for the DCs in the FBS-supplemented group.

Based on the results, HS-supplemented media may be the preferred option for DC culture (to replace FBS). Under the HS culture media, DCs were capable of maturation when induced with TNF-α, and showed high phagocytic ability and strong IL-12 gene expression. In addition, HS can be easily obtained and the culture process easily standardized. However, the PRP-supplemented medium was also shown to be suitable for DC culture as an alternative to FBS. PRP concentrations in media should be re-evaluated to achieve better results.

**Conclusion**

In conclusion, MNCs can be induced into DCs in media supplemented with HS or PRP instead of FBS. These DCs expressed CD86 and HLA-DR molecules, but not CD14. These DCs were also capable of phagocytosis and showed expression of IL-12. Thus, HS or PRP may be potential alternatives to FBS in the culture of DCs for clinical applications.
Abbreviations

CD: Cluster of Differentiation  
DC: Dendritic cells  
FBS: Fetal bovine serum  
HS: Human serum  
IL: Interleukin  
MNC: Mononuclear cells  
PRP: Platelet rich plasma  
TNF-alpha: Tumor necrosis factor alpha  
UCB: Umbilical cord blood

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Author Contribution

PQV: designed the experiment, performed the assays, analyzed the results, wrote the manuscript; NAT, PTHL: performed the experiments, NTS: designed, analyzed the results.

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