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Immunophenotypic Study of the Cord Blood CD34+ Progenitor Stem Cells-Derived Dendritic Cells

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Abstract:

Background: Dendritic cells (DCs) are bone marrow-derived cells of both lymphoid and myeloid stem cell origin that populate all lymphoid organs including the thymus, spleen, and lymph nodes, as well as nearly all no lymphoid tissues and organs. Although DCs are a moderately diverse set of cells, they all have potent antigen-presenting capacity for stimulating naive, memory, and effector T cells**.** DCs are receiving increasing scientific and clinical interest due to their key role in anti-cancer host responses and potential use as biological adjuvants in tumour vaccines, as well as their involvement in the immunobiology of tolerance and autoimmunity**.**

Objectives: To generate the dendritic cells (DCs) in vitro from purified cord blood stems progenitor cells and detects the growth curve of cells and analyze the DC in vitro differentiation pathway. Also to investigate the immunophenotypic CD markers morphology of the surface DC cells and to describe the effect of different growth factors on their expression.

Materials & Methods: For isolation and establishment in tissue culture of human DC, the cord blood was obtained from placenta of newly vaginaly delivered women, who were admitted in Al Kadhmyia Teaching Hospital. The cells were cultured in complete RPMI growth medium supplemented with growth factors GM-CSF+IL-4 for seven days then the key surface CD markers were detected by using the immunocytochemistry technique.

Results: The use of the CD34 monoclonal antibody in combination with CD45 monoclonal antibody has increased the opportunity of obtaining a reasonable amount of purified stem cells. Furthermore, the growth factors (GM-CSF+IL-4) that were supplemented to the complete medium played an important role in the differentiation of the CD34+ stem cells toward the DC cells. The growth factors GM-CSF and IL-4 when used in combination had made the difference and the rhythm of differentiation ,the count of cells, resolution of results, shape and size of cells, and the state of DC cells better than using each of them alone. The IL-4 when used in combination with GM-CSF act as inhibitory factor for the granulocyte and cells other than DC and at the same time has the capability to keep the DC cells in immature state. The kinetics of DC development in cord blood cultures was also determined. These results indicated that, the optimal culture of the cord blood-derived DC was 7-9 days. By using the immunocytochemistry technique, the key CD markers of DC cells has been revealed since the day 6 of culture. The immature DC cells were characterised with the positive expression of CD1a, CD11b and CD11c while the CD14 was negative.

Conclusion: Isolation, purification and mobilization of stem cells obtained were reliable and confirmatory, and indicate that the positive differentiation of the cord blood CD34+ stem cells toward the DC cells was achieved.

Key words; CD34 cord blood stem cells, immunophenotyping, dendritic cells

Introduction:

Dendritic cells (DCs) are bone marrow-derived cells of both lymphoid and myeloid stem cell origin that populate all lymphoid organs including the thymus, spleen, and lymph nodes, as well as nearly all nonlymphoid tissues and organs. Although DCs are a moderately diverse set of cells, they all have potent antigen-presenting capacity for stimulating naive, memory, and effector T cells [1, 2].

Dendritic cells originally identified by Steinman and his colleagues (1972) represent the pacemakers of the immune response. DCs are derived from bone marrow progenitors and circulate in the blood as

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immature precursors prior to migration into peripheral tissues. Within different tissues, DCs differentiate and become active in the taking up and processing of antigens (Ags), and their subsequent presentation on the cell surface linked to major histocompatibility (MHC) molecules [3].

Several studies have been carried out suggesting that there are different pathways for the formation of mature DCs from CD34+ or other primitive progenitors. Each pathway differs in terms of progenitors and intermediate stages, cytokine requirements, surface marker expression and, probably most importantly, biological function [4-7].

Sallusto and Lanzavecchia 1994 described the culture of human DCs from peripheral blood mononuclear cells in the presence of GM-CSF, GM-CSF+TNF- α, or GM-CSF+IL-4.

The phenotype and function of the cultured cells varied dramatically depending on the culture conditions. Cells cultured in GM-CSF+IL-4 were found to be the most potent APCs as measured by stimulation of the allogeneic mixed leukocyte reaction and priming of naive B lymphocyte against tetanus toxoid. However, IL-4 inhibits both granulocyte and macrophages development, seems to keep DCs in an immature state, thus more capable of processing exogenous antigens [8].

Materials & Methods

Culture of the cells:

The 50 samples of cord blood were obtained from placenta of newly delivered women, who were admitted in Al Kadhmyia Teaching Hospital for normal vaginal delivery. Cord blood samples were kept in 5 ml sterile heparinised plastic tubes and then brought to the stem cells culture laboratory to deal with it precisely.

Cord blood cell isolation and culture:

The CD34+ cells isolation and culture was done as described by Markowicz *et al.* [9], as follows, breafly: Fresh heparinised cord blood was diluted 1:1 in RPMI 1640 or PBS. The cell suspension was centrifuged in cooling centrifuge (at 4 $C^{o,}$ ^{2000} rpm for 10 min) after mixing with equal volume of Ficoll-Paque. Cells were washed three times in phosphate-buffered saline (PBS), and the cells were resuspended in the complete medium. Cells were counted using haemocytometer Neubaur chamber. Furthermore, the CD 34+, and CD45+ cells in suspension were detected by using anti CD34 and anti CD45 monoclonal antibody, respectively, by using immunocytochimistry technique [l0]. The complete growth medium which is used to culture DC cells was supplemented with 10% fetal calf serum (FCS), and with the specific growth factors that include human Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF 20 ng\ml) and Interleukin 4 (IL-4 20 ng\ml). Finally, the cells were cultured and incubated in CO2 incubator at 37 C^{o} for at least 7 days [11, 12, 13].

The comparison between different types of growth factors:

This method was used to determine the best growth factors that should be used in the culture of DCs. These factors were used under the same conditions and with the same specimen and were cultured at one time at the same concentration. The cord blood cells was isolated and cultured by same method that stated in the above cord blood isolation and culture section [9]. However, after washing three times with phosphate-buffered saline (PBS), the cells were resuspended with complete medium and they were divided into different tubes. The first tube contain complete medium supplemented with the GM-CSF (20ng\ml), the second one was supplemented with the IL-4 (20ng\ml), the third was supplemented with G-CSF (20ng\ml), the fourth was supplemented with

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GM-CSF (20ng\ml)and IL-4 (20ng\ml)while the final one was left without growth factors (complete medium alone) and considered as control.

The cells count was done by using trypan blue method; furthermore, the CD 34, and CD45 were detected by using anti CD34 and anti CD45 monoclonal antibody, respectively, by using immunocytochimistry technique [10]. The cells were cultured on the 96-wells micro titer plate in the presence of the complete medium supplemented with the growth factors that mentioned above. 10^6 cells were placed in each well. The plate was incubated at the CO2 incubator at the 36 C^o for at least 7 days. Examination of the cells culture was carried by using the inverted microscope and the cultures were refeeded with fresh complete growth medium and inspected every two days and when there was an 80% confluent, the culture was passaged and so on [11-13].

Kinetics of DC development in cord blood cultures:

The kinetics was investigated over an 11-day period. The cells from GM-CSF plus IL-4 supplemented cord blood cultures were harvested periodically over an 11-day period, counted, and analyzed for cell-surface phenotype. This kinetics was carried out as following: the cord blood cells was isolated and cultured by same method that stated in the above cord blood isolation and culture section [9]. However, after washing three times in phosphate-buffered saline (PBS), the cells were re-suspended in the complete medium. The cells was counted and the CD 34, CD45 were detected by using immunocytochimistry technique. The cells were cultured in the presence of the complete medium supplemented with the growth factors (GM-CSF 20 ng\ml and IL-4 20 ng\ml). 10^6 cells were placed in each well. The plate was incubated in the CO2 incubator at 37 C^o for at least 11 days. The cells were harvested on days 3, 5, 7, 9, and 11, respectively for enumeration of cells and were analyzed for the expression of the surface CD markers using the immunocytochimistry method. The surface markers include the CD1a, CD11c, and HLA-class II. These markers represent the key surface markers of the immature DCs.

Results:

The cells isolation and culture:

The optimal count of the cells that had been candidates for the cultivation was averaged between 10^6 to 2 X 10^6. The viability of the cells reached about 98% to 100%.

The results showed that, the cells were CD34+ and CD45+ in 65% and 75% respectively, figure (4-1).

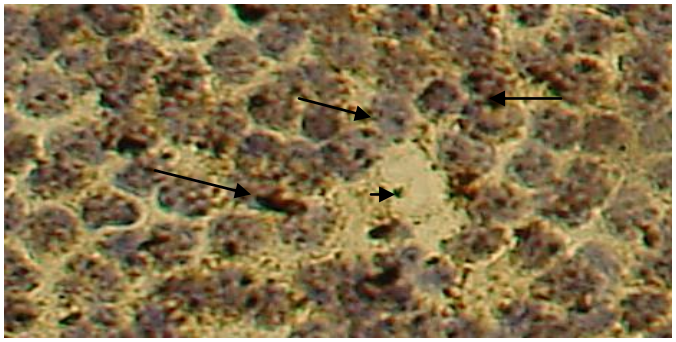

Figure (4-1): The Expression of cell surface CD34 marker that showed on the cord blood stem cells after purification of the cells, where the DAB (deep brown color) represents the positive cells while the hemotoxilin (blue color), the counter-stain, represents the negative cells(x160).

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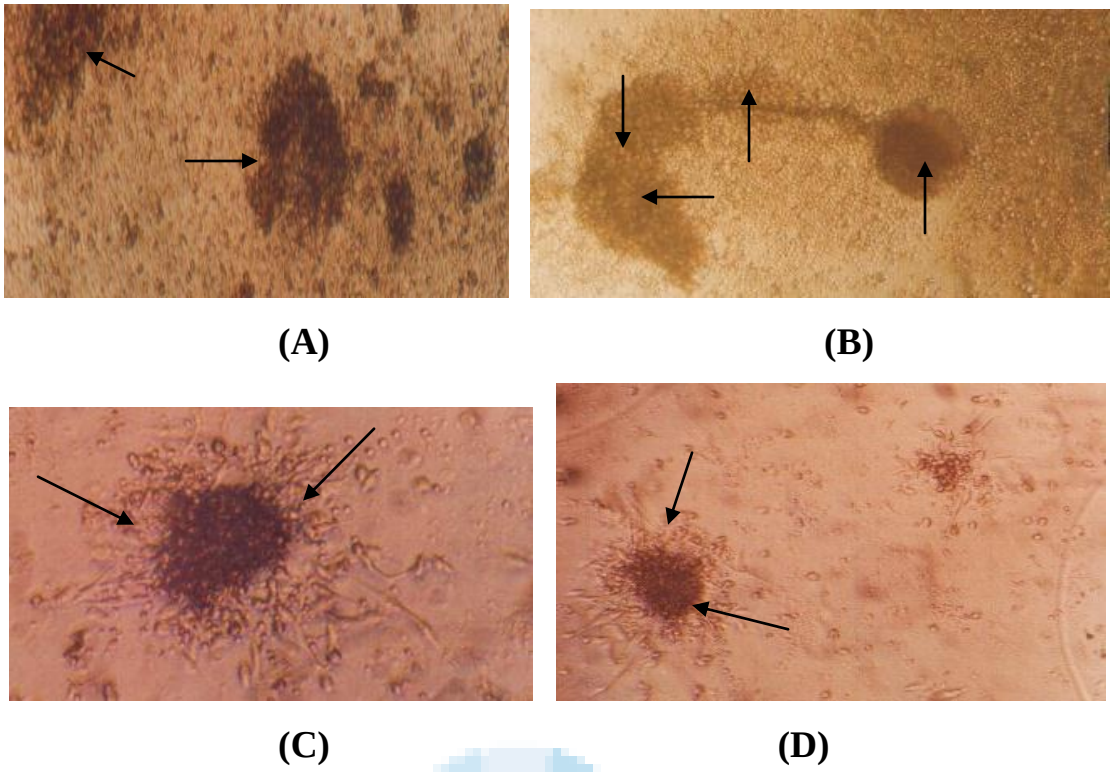

Figure (4-2): The morphology of the cells that cultured in the complete medium supplemented with 20ng/ml of GM-CSF plus 20 ng/ml of IL-4 and revealed by inverted microscope. (A) The cells after 5 day culture, note the colonies and clumps of the cells (x160). (B) Cells after 6 days culture that showed the clumps of the cells that arranged in straight line-like appearance(x160) (C) Cells after 7 days culture that showed typical characteristic dendritic cells, note the process (dendrites) that extend from the cells(x250).

(D) The cells after 7 days culture that showed the process (dendrites), which is the most important phenomenon of the DC cells that extend from the cells (x160)

The comparison between different types of growth factors:

The results are shown in **Table (4-1)** and Figure (4-4) as the following:

The cells cultivated with the complete medium supplemented with the 20 ng/ml of G-CSF were morphologically characterized with no signs of differentiation and there were no clumps or colonies of the cells. The dendrites which are the characteristic feature of the dendritic cells were not appeared. The cells count was not changed. After 7 days the key surface markers of DCs (CD1a, CD11c, CD11b, and HLA-DP, DQ, DR) were determined.

All of them were negative while the CD14 was positive. The cells enriched with the 20ng/ml of IL-4 factor characterized by there were few groups of clumps and colonies, there were no signs of differentiation but the cells count was raised. The CD14 was positive while the other key surface markers were negative.

The cells were also cultivated in media supplemented with the 20 ng/ml of the GM-CSF. Morphologically, the cells characterized with the

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presence of dendrites, significant differentiation appeared clearly and was characterized day by day, the cells were enlarged in the size and their shape and granules were budding accordingly. The cells count was $4X10^6$. The immunocytochimistry results were; the key surface markers of the immature DCs (CD1a, CD11c, CD11b, and HLA-class II) were positive, while the CD14 was negative. The cells that cultivated with both growth factors (20ng/ml of GM-CSF plus 20 ng/ml of IL-4) gave results resemble to the cells that were cultivated with GM-CSF alone. The only difference was that, the cells count was about $7X10^6$ and the rhythm of the cells differentiation was quicker.

Table (4-1): The comparison between different kinds of growth factors after cultivation of cells for 7 days.

Growth factors	Cells count	Surface CD markers					Morphology
		CD1a	CD11b	CD11c	CD14	HLA class II	
CM alone	106	-ve	-ve	-ve	+ve	-ve	No signs of differentiation
G-CSF	106	-ve	-ve	-ve	+ve	-ve	No signs of differentiation
IL-4	2X106	-ve	+ve	-ve	+ve	-ve	few groups of colonies, no signs of differentiation
GM-CSF	4X106	+ve	+ve	+ve	-ve	+ve	there was clumps and colonies of cells, signs of differentiation like dendrites and granules were appeared
GM-CSF + IL-4	7X106	+ve	+ve	+ve	-ve	+ve	The dendrites and granules were clearly appeared, the rhythm of development was very quick

The cells that were cultivated only in the complete medium (CM) (RPMI 1640 plus 10% FCS) were revealed no visible change in the shape or differentiation signs, no change in cells count **Figure (4-4).** The key surface markers were negative while the CD14 was positive. The most important phenomenon of differentiation was the dendrites that were appeared day by day and they appeared mostly in the clumps of cells. This phenomenon was revealed more and more especially in the third to fifth day of culture, Figure (4-3, C and D). The granules of cells witnessed increase in the size and changes in the shape and the numbers correlated with the date of cultivation. The cells were determined for the presence of the key surface markers of the DCs. These key markers include; CD1a, CD11c, CD11b, CD14, and HLA-classII; **figure (4-5).** The detection was carried out in the fifth to seventh day old of the culture. The result of the CD1a expression was positive in the all CD34+ cord blood specimens.

This marker was ranked first regarding the expression kinetics. In the most cases, the expression of the CD1a was revealed since the day 5 of the culture. The scores of the positive results of the CD11c expression were very high and satisfied in regarding to the other markers. The complete results of the surface DCs markers mentioned below in the **figure (4-5).**

The other surface CD markers were the CD11b and the CD14. The CD11b were positive in the most of cases (80%); whilst the CD14 expression was not more than 16%, **figure (4-5).**

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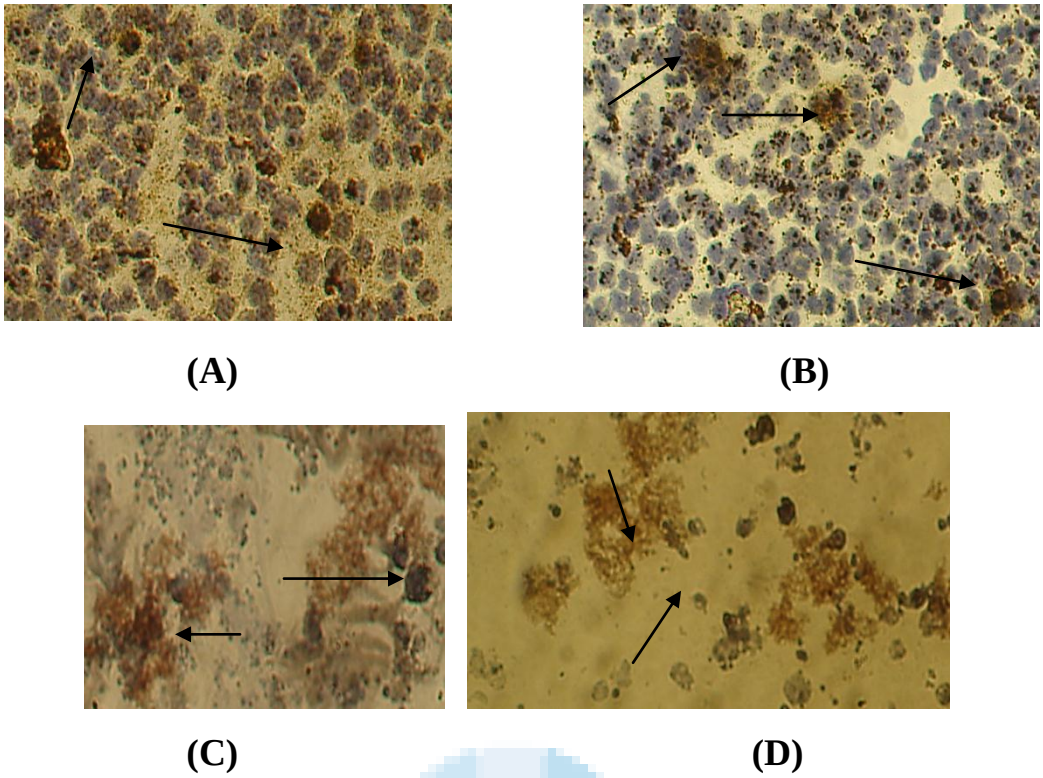
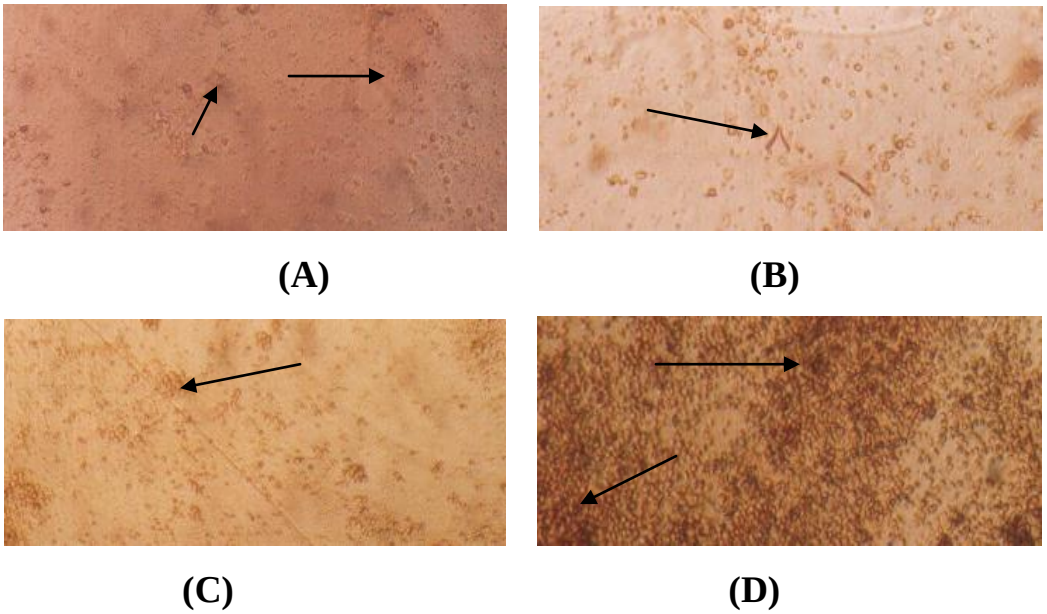


(A) **(B)**

(C) **(D)**

Figure (4-3): The Expression of cell surface markers that showed on the cells after 7 days culture in the complete medium supplemented with 20ng/ml of GM-CSF plus 20 ng/ml of IL-4, where the DAB (deep brown color) represents the positive cells while the hemotoxilin (blue color), the counterstain, represents the negative cells(x160). (A) Showing the expression of CD1a on the cells. (B) Showing the expression of CD11c on the cells. (C) Showing the expression of CD11b on the cells. (D) Showing the expression of HLA- class II on the cells.



(A) **(B)**

(C) **(D)**

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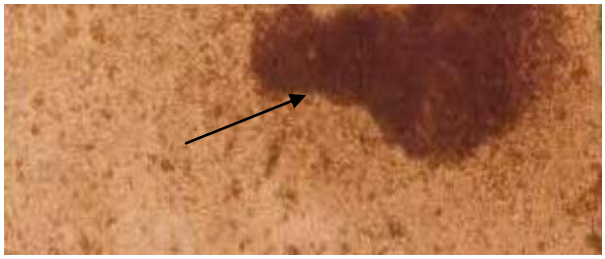

(E)

Figure (4-4) The comparison between different types of the growth factors after 7 days culture of the cells (A) The cells after 7 days culture in the complete medium alone (x160). (B) The cells after 7 days culture in the complete medium supplemented with 20 ng/ml of IL-4, (x160). (C) The cells after 7 days culture in the complete medium supplemented with 20 ng/ml of G-CSF (x160).

(D) The cells after 7 days culture in the complete medium supplemented with20 ng/ml of GM-CSF, (x160). (E) The cells after 7 days culture in the complete medium supplemented with 20ng/ml of GM-CSF plus 20 ng/ml of IL-4(x160).

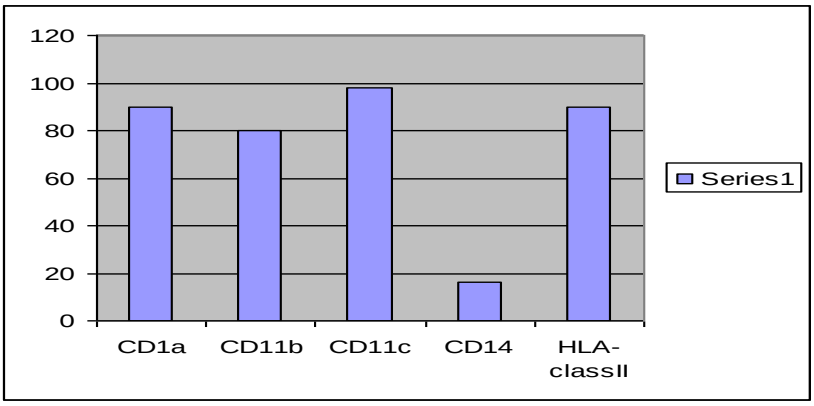

Figure (4-5): showed the results of the CD markers that had being done on the cord blood specimens. Each column represents the percentage of the certain CD marker that had been performed in the days 5 to 7 of the culture by using the immunocutochimistry technique.

Kinetics of DC development in cells cultures:

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Figures (4-6), (4-7) and Table (2) illustrates the readings of an 11-day culture as the following

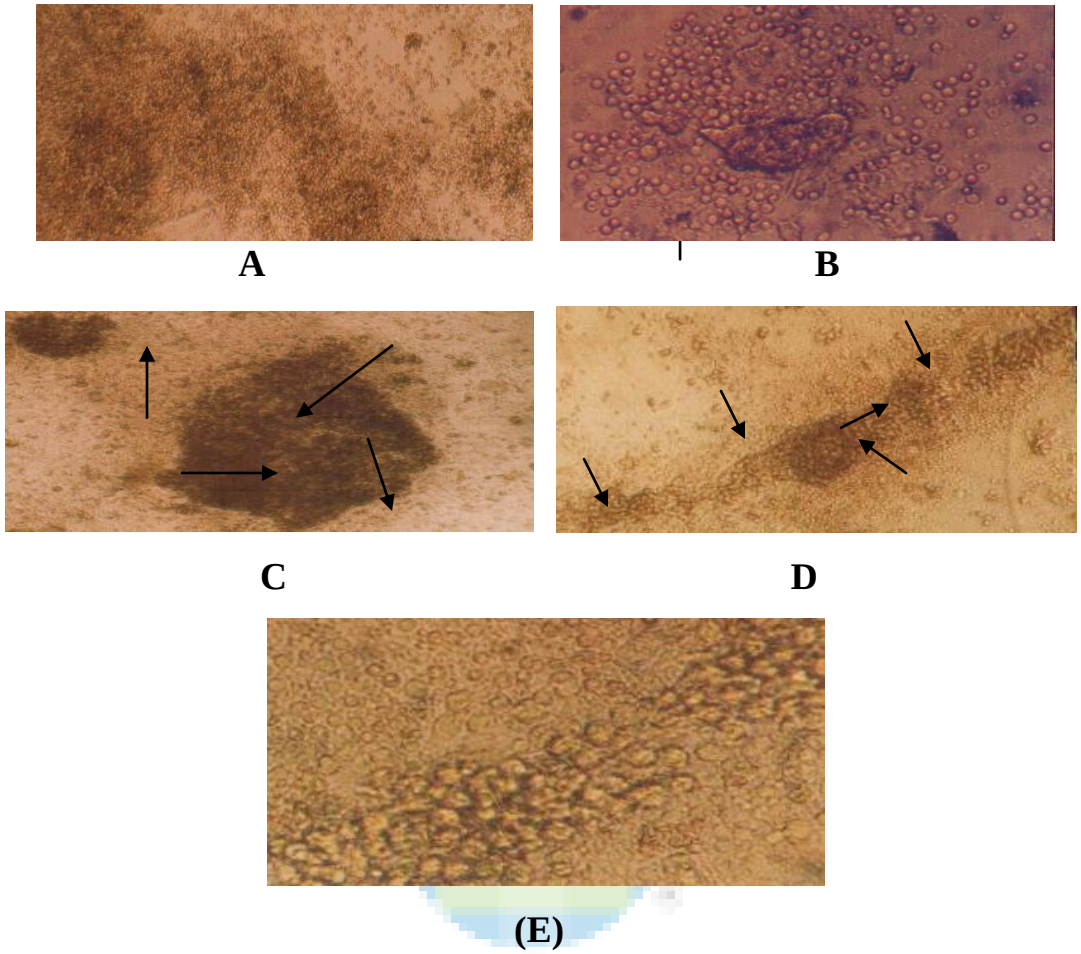

Figure (4-6): The kinetics of DC development from GM-CSF plus IL-4 supplemented cells cultures. Cells were cultured in $10^{[6]}$ cells/ml and on days 3, 5, 7, 9, and 11 of culture, were investigated. (A) The cells after 3 days culture(x160). (B) The cells after 5 days culture, note the process (dendrites) of the cells that showed in the middle of photo(x160). (C) The cells after 7 days culture, note the clumps and colonies of the cells(x160). (D) The cells after 9 days culture, note the clumps of the cells that arranged in the straight line-like appearance(x160). (E) The cells after 11 days culture, note the death of the cells (x250).

Table (4-2): Kinetics of DC development of an 11-day culture.

Day of the culture	Count of cells	CD markers expression	Remarks
Day 0	1x106	No expression	
Day 3	1x106	No expression	
Day 5	3x106	CD1a (90%) CD11c + HLA-II (30%)	
Day 7	3x106	CD1a (92%), CD11c (92%), HLA-II (80%)	
Day 9	3x106	CD1a (92%), CD11c (92%), HLA-II (80%)	
Day 11	2x106	CD1a (70%), CD11c (65%), HLA-II (60%)	Starting of cell death

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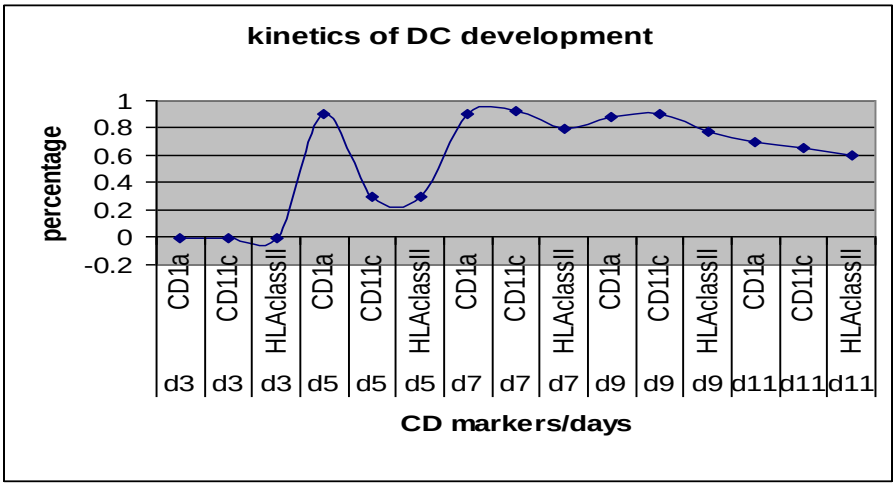

Figure (4-7): kinetics of DC development from GM-CSF plus IL-4 supplemented cord blood (CB) cultures. CB cells were cultured in 10^6 cells/ml and on days 3, 5, 7, 9, and 11 of culture, were harvested for enumeration of cells and analyzed for the expression of CD1a, CD11c, and HLA-class II.

Discussion:

In the present study the isolation and culture of human umbilical cord blood (CB) was done according to the collection of methods described by many investigators [6,7].

These techniques were performed in our lab for the first time in Iraq with some modifications according to the facilities available. The collection of samples was carried out in aseptic conditions and the cord blood was harvested within 24 h of collection period. It was observed that, any delay in the harvesting of cord blood could lead to decrease in the viability of the stem cells that was noticed by using the trypan blue method. The viability of the cells reached about 98% to 100%. This percentage dropped dramatically due to several reasons for example, the delay in culture of the specimens, the exposure to the direct sun light, the extreme high temperature, the extreme high speed of the centrifuge and the using of unsatisfied methods of purification.

The human umbilical cord blood was a rich source of the CD34 and recently it has been shown that CD45 was present in combination with CD34 on the cord blood derived stem cells [14]. Moreover, the cell count and the viability which were detected by trypan blue method were too satisfactory and reflect the success of this isolation method. This modified purification technique was based originally on the methods described by several workers [15, 16]. These results indicate that, the stem cells exist in high percentage in the cord blood harvest and regard a good sign to go further in the cultivation steps.

The cells were cultured with complete medium supplemented with specific growth factors (GM-CSF+IL-4). Most investigators used the RPMI 1640 medium plus 10% of foetal calf serum as a main source for tissue culture of DC cells [17, 18, 19]. This method was done according the protocol that described by [17, 18, 19, 20, 21]. Our observation revealed that, our cells gave the following results; CD1a+, CD11b+, CD11c+, and HLA-class II +. Whilst the CD14 was negative. Because there is no sole

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significant marker for the DC cells so far, in the present study cocktail of CD markers was used to determine markers present on the cultured cells. Recently the CD1a and the CD11c considered as the key surface markers that characterized the DC cells (16, 17, 19**)**; and some of them add the HLA-class II and combine it to the CD11c appearance [22].

The growth of cells revealed a monolayer within 24 h of culture. The number of cells that were included in the isolated colony varied and reach in some fields to about 15-20 cells. This criterion is regarded as one important feature of the IL-4 addition [15]. The most important phenomenon of differentiation was the dendrites that were appeared day by day and they appeared mostly in the clumps of cells. This phenomenon was revealed more especially in the third to fifth day of culture. The granules of cells witnessed increase in the size and changes in the shape and the numbers correlated with the date of cultivation. The dendrites and the granules were the most important features that characterized the DCs cells [1].

A combination 20 ng/ml of GM-CSF + 20 ng/ml of IL-4 gave better growth in comparison with the other used growth factors. The cells that cultivated with both growth factors (20ng/ml of GM-CSF plus 20 ng/ml of IL-4) gave results resemble to the cells that were cultivated with GM-CSF alone. The only difference was that, the cells count was about $7X10^6$ and the rhythm of the cells differentiation was quicker. These results indicated that, GM-CSF and IL-4 when used in combination they makes the difference and the rhythm of differentiation ,the count of cells, resolution of results, shape and size of cells, and the state of DC cells were better than from use each of them alone. The IL-4 when used in combination with GM-CSF act as inhibition factor for the granulocyte and cells other than DC and at the same time has capability to keep the DC cells in immature state. These results were in agreement with those reported by other investigators [8, 20, 23, 24].

The day 0 data were generated from freshly isolated cord blood cells. From the results that obtained we could draw the kinetics of the DC cells development through the observation of the change in the cells count and the demonstration of the surface CD markers that reflects the growth stages of DC cells. These results indicate that, the optimal culture of the cord blood-derived DC was 7-9 days regarding the cultivation conditions because the cells count reach constant level and the key surface CD markers were completed. Less or more than these periods could give false results (premature cells or starting of apoptosis). This data is agreed to those results described by Brasel *et al*. 2000 [21].

Conclusion:

Using the CD34 monoclonal antibody in combination with CD45 monoclonal antibody increase the opportunity of obtaining a reasonable amount of purified stem cells. Thus, this step was the key for other steps that followed. Furthermore, the growth factors (GM-CSF+IL-4) supplemented to the complete medium played an important role in the differentiation of the CD34+ stem cells toward the DC cells. The kinetics of DC development in cord blood cultures indicates that, the optimal culture of the cord blood-derived DC was 7-9 days. Less or more than this period could give false results (premature cells or starting of cells death). The growth factors GM-CSF and IL-4 when used together made the difference and the rhythm of differentiation , cell count , resolution of results, shape and size of cells, and the state of DC cells better than using each of them alone. By using the immunocytochemistry technique, the key CD markers of DC cells has been revealed since the day 6 of

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culture and the results obtained were reliable and confirmatory.

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