ORIGINAL ARTICLE

Peripheral blood monocytes can differentiate into efficient insulin-producing cells in vitro

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Abstract

Background: Recent studies provide evidence that peripheral blood monocytes have the ability to differentiate into mesenchymal-like cells. The ability of cultured monocytes to differentiate and produce insulin *in vitro* is analysed in the present study.

Methods: Peripheral blood monocytes were isolated from healthy donors and cultivated for fourteen days. Growth factors and liraglutide were used to induce pancreatic differentiation in most of the cultures. The growth factors were: monocyte colony-stimulating factor, interleukin-3, hepatocyte growth factor and epidermal growth factor. The rest of the cultures were cultivated only with nutrient medium and human serum. Insulin levels were measured by an enzyme-linked immunosorbent assay. Cellular morphology was observed using optical and electron microscopy. Cell membrane receptors were detected by flow cytometry.

Results: Monocytes were able to synthesize and excrete high levels of insulin after seven days in culture. A further increase in the excretion of insulin was observed after fourteen days. Cells were also able to differentiate and synthesize insulin, even if no growth factors were added to the culture medium. Some of the cultures were able to excrete insulin in a glucose-dependent manner. Differentiated monocytes were connected to neighbouring cells with axons and resembled the morphology of mesenchymal, dendritic and myeloid-progenitor cells. Cells retained their mature receptors and simultaneously developed immature receptors on their membrane.

Conclusions: Monocytes can acquire morphological properties of multipotent cells when they are cultivated under specific conditions *in vitro*. Differentiated monocytes are able to synthesize and excrete insulin.
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Keywords: Monocytes, mononuclear cells, insulin producing cells, diabetes, liraglutide, GLP-1, pancreatic beta cell differentiation

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Introduction

Monocytes have a very broad differentiation potential. They are not committed progeny of phagocytes (macrophages, Kuppfer cells, osteoclasts and microglial cells). They were able to differentiate *in vitro* to endothelial-like cells[1,2], osteoblast-like cells[3] dendritic-like, neural-like cells[4,5] and liver-like cells[6]. They differentiated to chondrocytes in the presence of Bone Morphogenic Protein-2 and -7 (BMP-2 and -7). They were inserted in the coronary arteries of the heart after a myocardial infarction and improved left ventricular function[7,8].

Circulating monocytes could be a source of cells which de-differentiate to mesenchymal cells. Monocytes cultivated *in vitro* in the presence of fibronectin, fetal bovine serum, and CD14-negative cells, acquired CD14, CD45, CD34 and type I collagen surface markers (CD marker = Cluster of Differentiation marker). Their morphology resembled endothelial and mesenchymal cells, and they were named Monocyte-derived Multipotent Cells (MOMCs). When the appropriate growth factors were added to the culture medium, MOMCs could express genes and morphological characteristics of osteoblasts, skeletal myoblasts, chondrocytes, lipocytes and endothelial cells. Probably CD14-negative cells excreted soluble factors that promoted monocytes' differentiation. Binding of monocytes to fibronectin was also essential for monocytes' differentiation[9,10].

Monocytes could be a source of insulin-producing cells due to their multipotency. There are only two studies so far which used monocytes as the source for insulin-producing cells[11,12]. Monocytes were de-differentiated *in vitro* for six days in the presence of Interleukin-3 (IL-3) and Macrophage Colony-stimulating Factor (M-CSF), a process that turned them to Progenitor Cells of Monocytic Origin (PCMOs). When PCMOs were further cultivated *in vitro* in the presence of Epidermal Growth Factor (EGF) and Hepatocyte Growth Factor (HGF) for 4-6 days, they were able to express pancreatic genes and produce small amounts of insulin. Implantation of these cells in the renal capsule of diabetic mice had a beneficial effect on hyperglycaemia control. The implanted cells were

rejected by the murine immune system after eight days[11]. M-CSF alone differentiates monocytes to macrophages. But when IL-3 is combined with M-CSF, monocytes become immature dendritic cells[13].

Glucagon-Like Peptide-1 (GLP-1) receptors influence the metabolism of monocytes. The incubation of monocytes with GLP-1 *in vitro* for 24 hours resulted in an increase of insulin receptors on the cells' surface. Furthermore, the binding of insulin on the surface of monocytes was increased[14]. Liraglutide is a synthetic analog of GLP-1 and has a stronger effect to monocytes. *In vivo*, the GLP-1 molecule's half-life is one hour while liraglutide's half-life is 11-15 hours. So it can affect the cells for a longer period of time[15]. Exendin-4 is another GLP-1 analogue and can affect the metabolism of mononuclear cells by reducing the pro-inflammatory responses. Possibly exendin-4 suppresses the mitogen-activated protein kinase (MAPK) signalling pathways[16].

Bone marrow mesenchymal cells from mice when cultivated *in vitro* with nicotinamide and exendin-4 for 14 days expressed genes of pancreatic tissue (*Pdx-1, Ngn3, NeuroD1, Pax-6* and *Glut-2*). The cells developed spheroid structures and produced almost 2.0 ng of insulin/10^6 cells. They could also increase their production by 1.5 fold in the presence of 450 mg/dl glucose. When these cells were transplanted in the liver of diabetic mice through the portal vein, the mice regulated their blood glucose levels during days 6 to 20. Cells were gradually rejected by the hosts' immune system[17].

Liraglutide directly affects monocytes. Exendin-4 promotes beta cell differentiation to murine bone marrow cells. It is challenging to combine these results. This is the first study that uses liraglutide (GLP-1 analogue) to monocytes, as a new factor for pancreatic beta cell differentiation. Additionally, another part of monocytes was grown only with serum and RPMI medium. Thereby, the sole impact of the plastic culture plate, RPMI, and human serum to monocytes and lymphocytes is studied for the first time.

Materials and Methods

Sample collection and handling

Peripheral vein blood was collected from healthy young adults, who were fasting for eight hours and did not take any medication. The mean age of the blood donors was 31 ± 4 years. Nine donors were male, and nine were female. The same blood sample was initially divided into four aliquots, as shown in Figure 1. All samples were cultured in a humidified incubator with 5% CO_2 at 37° C. From each cell culture, two samples were collected: one was collected at the standard glucose concentration of the supernatant 100 mg/dl (5.55 mmol/lit) and the second was collected after one-hour incubation at 250 mg/dl (13.88 mmol/lit) glucose concentration (Figure 1).

All blood donors were informed in detail, and they gave a written informed consent. All experiments were carried out in accordance with the principles of the Declaration of Helsinki as revised in 2008. The study was approved by the Bioethics and Ethics Committee of the Aristotle University of Thessaloniki (decision No A7246, 23-3-2011).

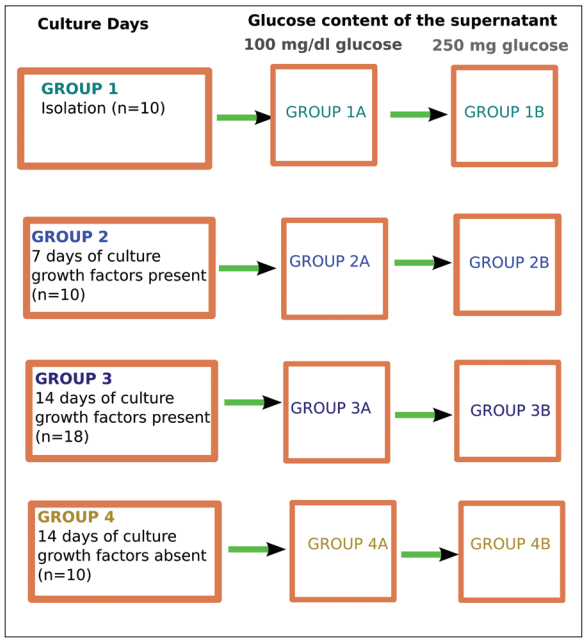

Figure 1: Diagrammatic presentation of the cell cultures depending on culture time and glucose concentration. Peripheral blood mononuclear cells from the same donor were divided into four aliquots and cultured in wells. Cells were tested for insulin production after: their isolation from the peripheral blood (Group 1), seven days in culture in the presence of growth factors (Group 2), 14 days in culture - growth factors present in the culture medium (Group 3), and 14 days in culture - growth factors absent in the culture medium (Group 4). The same cultures were tested for insulin excretion either at glucose concentration of 100 mg/dl (A) or 250 mg/dl (B) in the supernatant.

Mononuclear cells isolation

Approximately 15 ml of blood, diluted with 15 ml of RPMI medium and two ml of heparin, were placed on the top of 15 ml ficoll solution (1.077) in a 50 ml falcon tube, and then centrifuged at 400 g for 30 minutes at 30° C without brake. The white band (buffy coat), which contains the mononuclear cells (approx. six ml), was collected with a pipette, diluted with 24 ml RPMI and centrifuged in two 15 ml falcons at 300 g for 10 minutes at 30 °C without brake. The supernatant was discarded and the concentrated cells (1 ml x 2) were diluted with four ml RPMI. Cells were stained with trypan blue and counted in a Neubauer chamber. In all cases, they were approximately 12 x 10^6 and 99% of them were viable. Afterwards, cells were placed in a six-well plate, added one ml RPMI in each well and incubated for 1+1/2 hours. The non-adherent cells were washed away with RPMI.

Mononuclear cells' culture

The volume of each cell culture was two ml. Glucose concentration in culture medium was 100 mg/dl.

Monocytes cultivated in the presence of growth factors

(A) Cells were incubated with a de-differentiating medium for a period of seven days. Medium consisted of RPMI 1640, M-CSF (5 ng/ml), IL-3 (1 ng/ml), penicillin

(100 IU/ml), streptomycin 100 mg/ml, β-merkaptoethanol (10 μg/ml) and 10% human autologous serum. Half of the medium was replaced on day three.

(B) On the seventh day, the medium changed to the differentiating one, and the cells were further cultivated for another seven days. Differentiating medium consisted of RPMI 1640, EGF (10 ng/ml), HGF (20 ng/ml), penicillin (100 IU/ml) and streptomycin (100 mg/ml), nicotinamide (1 mg/ml), liraglutide (1 μg/ml) and human autologous serum (10%). Half of the medium was replaced on day 10.

Monocytes cultivated in the absence of growth factors

Cells were incubated only with RPMI and 10% autologous serum for 14 days. Half of the medium changed every three days.

All materials were supplied from Sigma-Aldrich (Taufkirchen, Germany), except for RPMI 1640 (Invitrogen, Paisley, United Kingdom) and liraglutide (Novo Nordisk, Bagsvaerd, Denmark).

Serum preparation

After ficoll centrifugation, the heparinized serum (five ml) was collected in a 15 ml falcon and centrifuged at 2000 g for 15 minutes at 24 ºC. The supernatant (serum) was filtered through Surfactant-Free Cellulose Acetate (SFCA) 0.20 μm filter and examined in a Neubauer chamber. No cells were detected in the serum. The isolated cells from one blood donor were fed only with serum from the same donor. So the serum is considered "autologous".

Microscopical analysis

Cell cultures were stained with May-Grünwald-Giemsa according to the manufacturer's instructions and examined under an Axio Observer inverted microscope (Carl Zeiss, Jena, Germany). Electron microscopy was performed as previously described[18].

Flow Cytometry

Flow cytometry was performed to collected cells on the first, seventh and 14th day and analysed for CD expression (Table 1). Cells were fully detached using a cell scraper and centrifuged at 400 g. The cell pellet was dissolved in two ml RPMI. Cell membrane receptors were detected in a Cytomics FC500 Flow Cytometer (Beckman Coulter, Milan, Italy). All CD antibodies were purchased from Exbio (Prague, Czech Republic), except for CD209 (Biolegend, San Diego, USA). Flow cytometry was performed according to the manufacturer's instructions and analysed with the freeware "Flowing Software" version 2.5.1 (Perttu Terho, Turku, Finland).

Table 1: CD markers used in flow cytometry in the study.

Cluster of differentiation	Characteristic marker
CD45	Every white cell
CD14	Monocytes
CD16	Macrophages
CD33	Colony Forming Unit- Monocytes
CD34	Colony Forming Unit- Granulocytes and Monocytes
CD209	Dendritic cells

CD: Cluster of Differentiation.

Insulin measurements

Cell cultures were tested for insulin production on the first, seventh and 14th day. An aliquot of 50 μl from the supernatant was collected, when the culture medium had the initial glucose concentration of 100 mg/dl. The rest of the cultures' supernatant was preserved and extra glucose was added (approximately 50 μl) to reach the final glucose concentration of 250 mg/dl. After one-hour incubation at 250 mg/dl glucose, the supernatant was completely removed and stored in a deep freezer (-75 ºC). Insulin levels were measured by the *Human* Insulin ELISA Kit KAQ1251 (Invitrogen, Paisley, United Kingdom) according to the manufacturer's instructions.

Statistical analysis

The Statistical Package for the Social Sciences (SPSS) version 21 (IBM Corp., New York, USA) was used for sample analysis. Initially, the samples in each group were checked for normality. Since each group had a number of samples smaller than 50, the Shapiro-Wilk test was used. Group 3 was compared with non-parametric tests because it did not have a normal distribution ($p = 0.012$). Other groups were compared with parametric tests because they had normal distributions. The level of statistical significance was set for $p < 0.05$.

Results

Flow cytometry

The most characteristic CD marker of each cell type is shown in Table 1. CD33 and CD34 correspond to monocyte progenitor cells while CD16 and CD209 correspond to monocyte descendants.

The percentage of monocytes in the culture gradually increased (Table 2). The mean percentage of monocytes (CD14 and CD45 positive) cells at the beginning of the cultures was 13.64 ± 4.04 % [mean ± standard deviation (SD)]. The remaining cells were lymphocytes. On the seventh day, CD14 positive cells had risen to 78.69 ± 11.69 %, because the floating lymphocytes were gradually discarded from the supernatant. Lymphocytes did not adhere to culture plastics, and they floated freely in the supernatant. So lymphocytes were reduced every time the supernatant was replaced. On the 14th day the percentage of CD14 positive cells was 66.08 ± 10.74 %.

The changes of the CD markers on the monocytes' surface according to the culture duration are shown in Table 2. Monocytes on the first day of the cultures already expressed both markers of maturity CD16 48.79 ± 13.46 % and of immaturity CD34 7.27 ± 3.23 % (mean ± SD). When growth factors were present, on the seventh day the monocytes highly expressed CD209 88.77 ± 11.5 %, which corresponds to dendritic cells, and CD16 97.74 ± 2.74 %, which corresponds to macrophages. Also, they expressed

CD33 88.16 ± 13.01 % and CD34 68.53 ± 14.34 %, which are immature markers. So, monocytes were evolving to cells that expressed simultaneously mature and immature membrane receptors. On the 14th day, differentiated monocytes maintained their maturity markers (CD209 79.02 ± 11.07 % and CD16 91.18 ± 10.28 %) and reduced their immaturity markers (CD33 76.80 ± 16.36 % and CD34 55.96 ± 18.99 %). So the cells were gradually losing their potency.

Monocytes cultivated without growth factors in the culture supernatant evolved more slowly to the same CD pattern. On the 14th day, almost all of the CD14 positive cells expressed CD209 95.1 ± 1.41 %, CD16 99.15 ± 0.31 and CD33 96.46 ± 0.36. They also expressed the highest levels of CD34 which is 76.79 ± 9.05 %.

Lymphocytes evolved to a very different CD pattern (Table 3). After the isolation, 9.79 ± 3.27 % of the lymphocytes expressed the CD16 marker, which corresponds to the Natural Killer cell marker. Cultivated in the presence of growth factors, the lymphocytes expressed CD16 marker on the seventh day 62.33 ± 12.70 % and on the 14th day 33.45 ± 8.79 %. Cultivated in the absence of growth factors, lymphocytes expressed CD16 marker 39.47 ± 16.09 % and 47.85 ± 15.18 % respectively.

Insulin measurements

ELISA results are shown in Table 4. On day one, there was no insulin in the supernatant (not detected). No insulin was detected even after the addition of extra glucose in the supernatant of the cell cultures on day one.

Mononuclear cells which were cultivated in the presence of growth factors for seven days, produced 10.32 ± 6.78 μU (mean ± SD) insulin. When the culture period was extended to 14 days, they produced on average 17.03 ± 12.76 μU. The mean production was 70% increased by the extension of the culture period. Afterwards, the addition of extra glucose in the supernatant caused mixed effects. Some cultures excreted insulin and other cultures bound insulin to membrane-receptors. This phenomenon shows an unstable biological answer to the raised glucose levels. It is noteworthy that changes to insulin excretion vary considerably among the cultures (Table 4).

When cells were cultivated in the absence of growth factors for 14 days, they produced on average 15.05 ± 6.2 μU (mean ± SD) insulin. It is about 22 % less than the average production of the cultures with growth factors. After the addition of extra glucose, all cultures increased the production of insulin. The mean production was 22.81 ± 6.02 μU per well. These results show a pure biological phenomenon, which was steadily repeated in all experiments. Insulin production was regulated according to the glucose levels. The mean increase of insulin in one hour was 7.76 μU (0.28 ng) per 10^5 cells (Table 4).

Statistical comparison of insulin production between groups

Insulin production varied among the groups. Additionally, the response of cells to the change of supernatant glucose concentration from 100 mg/dl to 250 mg/dl varied considerably among the groups (Table 4). Statistical analysis was used to reveal if these differences are statistically significant. Groups with the letter A had supernatant glucose concentration of 100 mg/dl while groups with letter B had supernatant glucose concentration of 250 mg/dl. Results are shown in Table 5.

1. (i) Group 2 was cultivated for seven days in the presence of growth factors. Group 2B did not change its insulin production compared with group 2A (p =0.920). So, the cells were not able to increase insulin production in response to the increased glucose concentration.

(ii) Group 3 was cultivated for 14 days in the presence of growth factors. Group 3B produced more insulin (19.45 ± 9.00 μU) than group 3A (17.03 ± 12.76 μU) (mean±SD), but the result is not statistically significant (p =0.492). Again cells do not excrete insulin in a glucose-dependent manner.

Table 2: Mean CD expression of the cultured monocytes ± standard deviation (CD45+ and CD14+).

Days in culture - presence of GFs	Day 1 Isolation (n =3)	Day 7 GFs present (n =10)	Day 14 GFs present (n =9)	Day 7 GFs absent (n =3)	Day 14 GFs absent (n =3)
Monocyte % of CD45+ cells	13.64 ± 4.04	78.69 ± 11.69	66.08 ± 10.74	70.90 ± 7.19	79.63 ± 6.91
CD209	13.95 ± 4.76	88.77 ± 11.5	79.02 ± 11.07	75.87 ± 9.54	95.10 ± 1.41
CD16	48.79 ± 13.46	97.74 ± 2.74	91.18 ± 10.28	85.53 ± 6.49	99.15 ± 0.31
CD33	60.02 ± 2.12	88.16 ± 13.01	76.80 ± 16.36	66.41 ± 2.04	96.46 ± 0.36
CD34	7.27 ± 3.23	68.53 ± 14.34	55.96 ± 18.99	35.3 ± 16.12	76.79 ± 9.05

CD: Cluster of Differentiation, n: number, GFs: growth factors.

Table 3: Mean CD expression of the cultured lymphocytes ± standard deviation (CD45+ and CD14-).

Days in culture - presence of GFs	Day 1 Isolation (n =3)	Day 7 GFs present (n =10)	Day 14 GFs present (n =9)	Day 7 GFs absent (n =3)	Day 14 GFs absent (n =3)
Lymphocyte % of CD45+ cells	86.36 ± 4.04	21.31 ± 11.69	33.92 ± 10.74	29.1 ± 7.19	20.38 ± 6.91
CD209	0.05 ± 0.04	4.98 ± 6.43	4.32 ± 4.45	0.99 ± 1.72	0.41 ± 0.58
CD16	9.79 ± 3.27	62.33 ± 12.70	33.45 ± 8.79	39.47 ± 16.09	47.85 ±1 5.18
CD33	1.07 ± 0.16	10.84 ± 8,75	5.15 ± 8.35	1.47 ± 0.73	1.67 ± 1.15
CD34	0.01 ± 0.01	1.52 ± 2.47	1.05 ± 2.65	0.00 ± 0.0	0.00 ± 0.0

CD: Cluster of Differentiation, n: number, GFs: growth factors.

Table 4: Supernatant insulin content of the cultured wells (volume 2 ml – cell number 10^5 / well).

Culture duration and group	Day 1 - Group 1 Isolation day		Day 7 - Group 2 Presence of growth factors		Day 14 - Group 3 Presence of growth factors		Day 14 - Group 4 Absence of growth factors	
Glucose concentration	**100 mg/dl**	**250 mg/dl**	**100 mg/dl**	**250 mg/dl**	**100 mg/dl**	**250 mg/dl**	**100 mg/dl**	**250 mg/dl**
Sample 1	nd	nd	20.32	11.68	27.54	14.87	24.74	30.86
Sample 2	nd	nd	8.50	4.78	53.81	37.54	10.88	24.74
Sample 3	nd	nd	5.48	2.27	31.51	29.56	18.79	28.90
Sample 4	nd	nd	6.96	10.08	4.78	21.08	16.46	21.08
Sample 5	nd	nd	8.50	9.29	8.50	32.14	14.08	23.30
Sample 6	nd	nd	0.89	6.21	7.73	7.73	25.46	27.54
Sample 7	nd	nd	4.09	6.21	30.21	13.28	10.08	23.30
Sample 8	nd	nd	10.88	26.16	23.30	24.02	9.28	18.02
Sample 9	nd	nd	19.56	10.88	12.48	6.21	8.48	9.91
Sample 10	nd	nd	18.02	13.28	15.25	27.39	12.25	20.44
Sample 11					16.6	20.50		
Sample 12					7.97	14.92		
Sample 13					3.16	12.58		
Sample 14					10.93	17.60		
Sample 15					15.91	11.59		
Sample 16					22.88	10.58		
Sample 17					7.61	18.27		
Sample 18					6.41	30.16		
Mean ± SD	not detected	not detected	10.32±6.78	10.08±6.60	17.03±12.76	19.45±9.00	15.05±6.20	22.81±6.02
	Group 1A	**Group 1B**	**Group 2A**	**Group 2B**	**Group 3A**	**Group 3B**	**Group 4A**	**Group 4B**

SD: standard deviation.

(iii) Group 4 was cultivated for 14 days in the absence of growth factors. Group 4B was the only group that could significantly increase the production of insulin compared with group 4A ($p < 0.001$). Cells regulated insulin production according to the glucose levels and produced significantly more insulin when the glucose concentration of the supernatant rose from 100 mg/dl to 250 mg/dl.

2. The difference between the group 2A (seven-day culture) and 3A (14-day culture) is not statistically significant ($p = 0.191$). So the difference in culture duration and medium didn't statistically influence the production of insulin at 100 mg/dl glucose.

3. The difference between the group 2B (seven-day culture) and 3B (14-day culture) is statistically significant ($p = 0.008$). So the culture duration and the medium change enhanced significantly the production of insulin at 250 mg/dl glucose.

4. The production of insulin was not significantly different between the group 3A, cultivated in the presence of growth factors in the culture medium, and the group 4A, cultivated in the absence of growth factors in the culture medium ($p = 0.811$). So, growth factors and liraglutide neither were necessary for cellular differentiation to insulin-producing cells nor statistically increased insulin production.

Morphological analysis of the cells

In the beginning of the cultures, all cells had small round shape. Monocytes and lymphocytes were indistinguishable (Figure 2). As the culture days passed by, monocytes enlarged and some of them developed axons. After 14 days, their shape became irregular, and they looked like mesenchymal, dendritic and myeloid-progenitor cells (Figure 3 and Figure 4). Gradually they started

Table 5: Statistical comparisons of the corresponding groups according to research questions.

Question	Group comparison	Statistics	p value
1. Do cells produce more insulin when the supernatant glucose concentration changes from 100 to 250 mg/dl?	(i) 2 A and 2 B	Parametric. Paired samples t test	0.920
	(ii) 3 A and 3 B	Non parametric. Wilcoxon signed ranks test	0.492
	(iii) 4 A and 4 B	Parametric. Paired samples t test	0.000
2. Does culture time extension from 7 to 14 days and medium change influence the amount of insulin production at 100 mg/dl glucose?	2 A and 3 A	Non parametric. Mann-Whitney test for two independent samples	0.191
3. Does culture time extension from 7 to 14 days and medium change affect the amount of insulin production at 250 mg/dl glucose?	2 B and 3 B	Parametric. Two independent samples t test for equal variances (Levene test for equality of variances p=0.112)	0.008
4. Does the presence of growth factors in the supernatant affect the production of insulin?	3 A and 4 A	Non parametric. Mann-Whitney test for two independent samples	0.811

to connect to the neighbouring cells with various ways. They developed membrane-to-membrane connections, axons or cytoplasmic fusion (Figure 3, Figure 4 and Figure 5). Some cells formed double nuclei (Figure 5).

After 14 days in culture in the presence of growth factors, differentiated monocytes were observed under the electron microscope. The cells connect to each other firmly with membrane gap and tight junctions (Figure 6). Gap junctions are a form of a synapse. Cells had various morphologies, so they probably had also various roles in the culture.

On the contrary, lymphocytes retained their size and round shape. They did not change morphologically (Figure 3). They either floated in the supernatant of the culture, or they touched the monocytes' membrane. They did not form axons. Their cellular connections were only achieved by sticking their membrane to the monocytes' membrane.

Discussion

This is the first study that uses liraglutide, a human long-acting agonist of the GLP-1 receptor, in order to enhance the production of insulin from monocytes. It is the second study that differentiates monocytes to insulin-producing cells, and it is the first to optimize their production. A strong point of the study is that the experiments were performed under strictly defined conditions, and the results were reproducible with every donor. Also, the study was performed *in vitro*, and this proves that only mononuclear cells are responsible for all the results.

Monocytes adhere to culture plastics, change their surface markers, size, and morphology. All the monocytes' CD expressions were as high as CD14 expression. This proportional change of markers with CD14 implies that the differentiated cells have a monocytic origin.

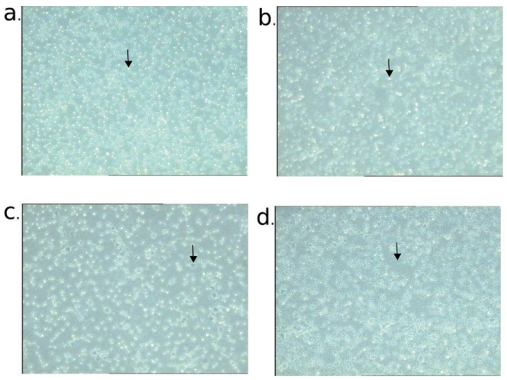

Figure 2: Morphology of the isolated monocytes and lymphocytes on the first day. The black arrows mark one characteristic cell in each picture. a) After isolation, monocytes and lymphocytes were small round cells. b) Afterwards, cells gradually changed shape and developed ellipses. This is the first step to the making of axons. At the same time, they started to attach to the bottom of the well. c) Some cells had larger and darker nucleus and cytoplasm from the first day of the culture. d) Also there were some platelets in the culture, despite the ficoll centrifugation. Some cells were destroyed during the centrifugation process and the cellular debris are evident in the culture well. Platelets and debris reduced each time the supernatant was replaced. Cells were not stained, magnification: 20x.

Monocytes can obtain immature surface markers and at the same time preserve their initial identity markers. The CD14 expression increases linearly with the expression of the other CD markers, both mature and immature. Metaphorically, monocytes are "stretched" backward and forward to their differentiation pathway. Monocytes differentiate and develop cellular connections and structures, like trying to create a tissue. Small vesicles in the cytoplasm of the cells after 14 days of culture contain probably insulin (Figure 6b). On the other hand, lymphocytes retain their morphology.

Cells cultivated in the presence of growth factors were not able to regulate insulin excretion according to the glucose level of the supernatant. This phenomenon implies that differentiated monocytes have not become pancreatic cells. The variation in insulin-excretion among the cultures could reflect a different stage of culture differentiation.

On the other hand, cells cultivated in the absence of growth factors were able to regulate insulin excretion according to the glucose level of the supernatant. So, cells have acquired some of the properties of pancreatic cells. There is not much variation in insulin-excretion among

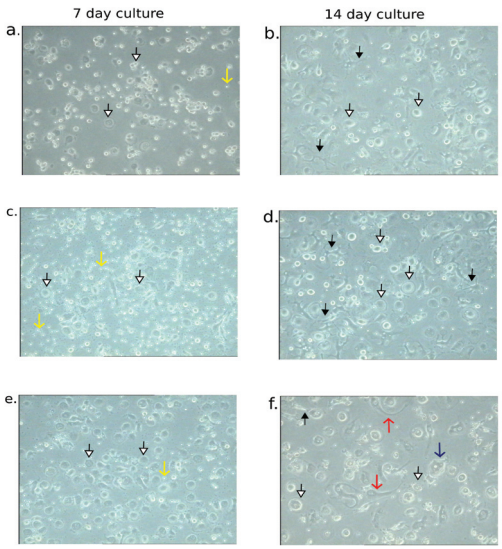

Figure 3: Morphological changes of the cultivated mononuclear cells observed by optical microscopy**.** Monocytes constantly changed their morphology and their size during the whole culture period. Lymphocytes remained unchanged. The white arrows show monocytes which enlarged on day seven and they further enlarged on day 14. Their morphology resembled the morphology of myeloid-progenitor cells. Both the nucleus and the cytoplasm became much bigger in comparison with Figure 2. Their morphology resembled the morphology of myeloid-progenitor cells. The black arrows show some cells which exhibited the morphology of dendritic cells on day 14. The red arrows mark cells with morphology of mesenchymal cells. The blue arrow shows a cell which resembled the morphology of myeloblast. The yellow arrows mark the lymphocytes which remained morphologically unchanged. The density of the cells varied on day seven (a), (c), but it became similar on day 14 (b), (d). Cells in pictures (a), (b) (c), (d) were cultured in the presence of the growth factors and cells in pictures (e), (f) were cultured in the absence of the growth factors. Cells were not stained, magnification: 20x.

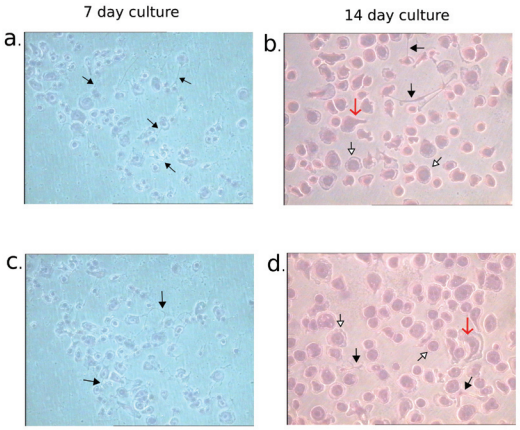

Figure 4: Optical microscopy images of cell cultures stained by May-Grünwald-Giemsa. When the cells were stained, nuclei enlargement and cellular connections (black arrows) became more obvious. Cellular axons are thinner on day seven in comparison with day 14. The black arrows on day 14 mark the developed cellular axons. The morphology of axon cells resembles the dendritic cells. The white arrows show cells with progenitor morphology. The red arrows show cells with mesenchymal morphology. Cells in pictures (a) and (b) are grown in the presence of growth factors and in (c) and (d) in the absence of them. Magnification: 20x.

the cultures. Therefore, cultures have reached the same stage of differentiation.

Insulin production and excretion are probably spontaneous as the cells obtain immature characteristics. Probably cells use insulin as a growth factor for themselves. Differentiated monocytes become immature cells that have unlocked progenitor genes. They possibly use insulin for increasing their potency. Exogenous growth factors are not necessary for differentiation and insulin excretion. This finding is further supported from a recent study[19]. However, there is a trend towards more insulin synthesis in the presence of growth factors and liraglutide, at 100 mg/dl glucose concentration. Probably there would be a significant difference in insulin synthesis if the cultures' duration expanded beyond 14 days.

The culture environment, the platelets and the lymphocytes are helpful for monocyte differentiation. *In vivo*, monocytes and lymphocytes cooperate and act as a team, so the immune system functions properly. Similar cooperation is achieved *in vitro* and it seems that lymphocytes support monocyte differentiation. Possibly, the CD16 positive lymphocytes' role is to activate the adherent monocytes. Cultures also contain a small amount of platelets. It seems that activated platelets help monocytes to adhere to well plastics[20]. Platelets do not produce insulin. Instead, they consume insulin through their GLUT3 glucose-receptors[21] and they reduce the insulin content of the supernatant. In conclusion, every cell in the culture interacts with the neighbouring and together they finely regulate their micro-environment in a unique way. They produce a lot of proteins to achieve their differentiation, which are still unknown.

Monocytes can differentiate to cells that produce adequate amount of insulin to stabilize blood glucose. The mean excretion of insulin in one hour *in vitro* was 7.76 μU

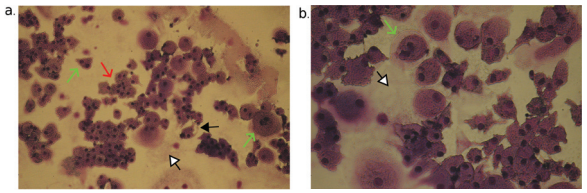

Figure 5: Optical microscopy images of cell cultures stained by May-Grünwald-Giemsa on the 14th day. Cells connect to each other with membrane-membrane junctions (red arrow). Some of them develop axons (black arrow). A new type of connection is revealed in this magnification. Cells attach firmly to the bottom of the well, spread their cytoplasmic structures and fuse them together (white arrows). Also cells with two nuclei are revealed (green arrows). Magnification: 40x.

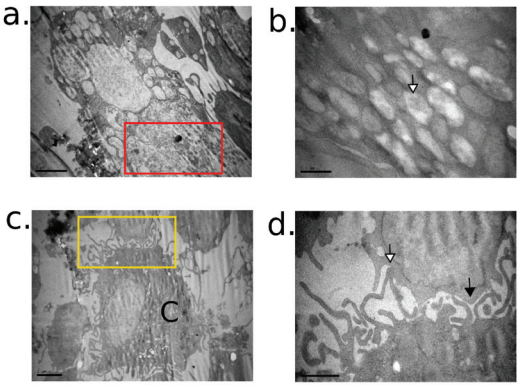

Figure 6: Electron microscopy images of differentiated monocytes after 14 days in culture in the presence of growth factors. a) Differentiated monocytes contain many storing granules (red rectangle). Magnification 10.000x. b) Granules are filled with proteins (white arrow). Magnification 40.000x. c) Monocytic origin cell (C) extends large axons. Magnification 8.000x. d) The yellow rectangle of (c) is magnified. The central cell (C) connects to neighboring cells with gap junctions (black arrow) and tight junctions (white arrow). Magnification 25.000x.

or 0.28 ng per 10^5 cells. A small number of cells (10^5) can produce an adequate amount of insulin. For comparison *in vivo*, a human with 5 litres of blood in a fasting state has 22.8 μU or 0.912 ng of insulin in his bloodstream[22]. So, $4x10^5$ cells could theoretically produce enough insulin to cover the fasting need of an adult. Mononuclear cells could be candidates for cell replacement therapy of diabetes in the future.

Cell replacement therapies provide new therapeutic tools for diabetes, as they aim to regenerate beta cell mass. The replacement of pancreatic beta cells with adult or embryonic stem cells is of particular interest today. However, these cells are rare *in vivo*. It is difficult to obtain and to cultivate them. It is difficult to maintain their potency and proliferation when they are cultivated *in vitro*. Their cryopreservation is expensive[23,24]. Embryonic stem cells are able to cause tumors[25]. Adult stem cells are safe for use in vivo[26-29].

Monocytes could be used for islet regeneration therapies as autologous transplants. A major challenge is to differentiate autologous human adult cells to insulin-producing cells *in vitro* and then transplant them back into the body. This autologous transplantation excludes an immune

rejection. Peripheral blood mononuclear cells are readily available in large numbers. They do not need storage in cell banks because they are always available. They do not pose ethical issues like the embryonic stem cells. Differentiated monocytes resemble the adult stem cells.

Monocytes have potency and plasticity. Maybe monocytes leave the bone marrow and scatter in the body to participate in the repair of the tissues. Possibly, they have the same role with the seeds of the plants. Mononuclear cells have the remarkable ability to generate CD34 positive cells, and they could be a source of multipotent cells. On the other hand, dysfunction of the immune system is responsible for many autoimmune diseases. So the use of differentiated monocytes in humans is not safe yet. It should be clear if differentiated monocytes can cause autoimmune disease or not. Animal experiments are required to test the safety of these cells.

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Conflict of Interest

The authors have declared that no competing interests exist.

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