Transformation of Adult Mesenchymal Stem Cells Isolated From the Fatty Tissue Into Cardiomyocytes

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Background. **Myocardial infarction results in the death of cardiomyocytes, which are replaced by scar tissue. Cardiomyocytes cannot regenerate because they are terminally differentiated. Mesenchymal cells are pluripotent cells, which have the potential to differentiate to specialized tissues under appropriate stimuli. The aim of this study was to direct differentiation of the adult mesenchymal stem cells isolated from fatty tissue into cardiomyocytes using 5-azacytidine.**

Methods. **Adult mesenchymal stem cells were isolated from the fatty tissue of New Zealand White rabbits and cultured in RPMI medium. Second-passaged mesenchymal cells were treated with various concentrations of 5-azacytidine and incubated for different intervals of time. The cells were plated in six-well dishes at 500, 5,000, and 50,000 cells/well. These cells were treated with 1-, 3-, 6-, 9-, and 12-μmol/L concentrations of 5-azacytidine and incubated for 12, 24, 48, and 72 hours. Later, the medium was replaced with fresh medium and incubated in a CO_2 incubator. The medium was changed once at 3 to 4 days. At 2 months, the cells were fixed with 0.4% glutaraldehyde for 2 hours and later washed with phosphate-buffered saline. The transformed cells were subjected to immunostaining for the myosin heavy chain, α actinin, and troponin-I.**

Results. **After treatment with 5-azacytidine, the adult mesenchymal stem cells were transformed into cardiomyocytes. At 1 week, some cells showed binucleation and extended cytoplasmic processes with adjacent cells. At 2 weeks, 20% to 30% of the cells increased in size and formed a ball-like appearance. At 3 weeks, these cells began to beat spontaneously in culture when observed under phase contrast microscope. Immunostaining of the transformed cells for myosin heavy chain, α actinin, and troponin-I was positive. The differentiated cells maintained the phenotype and did not dedifferentiate up to 2 months after treatment with 5-azacytidine.**

Conclusions. **These observations confirm that adult mesenchymal stem cells isolated from fatty tissue can be chemically transformed into cardiomyocytes. This can potentially be a source of autologous cells for myocardial repair.**

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Myocardial infarction is a leading cause of mortality and morbidity. Myocardial infarction results in the death of cardiomyocytes. Cardiomyocytes do not regenerate after birth; they undergo hypertrophy but not hyperplasia. But recently, Anversa and associates [1] have shown the presence of cardiogenic stem cells residing in the heart, which is encouraging, but their number is limited. Due to the paucity of cardiogenic stem cells in the heart native, this may not be a therapeutic option for treating heart failure. Hence, cell transplantation could be an alternative treatment modality for cardiac regeneration.

Recent studies have shown that if cardiomyocytes are transplanted into scar tissue [2], these transplanted cells limit the scar expansion and prevent postinfarction heart failure. The transplantation of cultured cells into damaged myocardium has been proposed as a treatment for heart failure. To study cardiac myocyte development, different approaches have been established. Studies have been done to find potential source of cells for myocardial repair. Fetal cardiomyocytes [3–7], cell lines [8], skeletal myoblasts [9], smooth muscle cells [10], fibroblasts [11], and bone marrow cells [12, 13] have successfully been transplanted into normal and infarcted myocardium. All these transplanted cells survived initially but were slowly eliminated due to immune rejection. We chose the adult mesenchymal stem cells (MSC) because they can be self-renewed in an undifferentiated state and can be directed to differentiate to the cell type of choice and auto- transplanted. Recent published reports have revealed that these MSC can be differentiated into various cell types, including bone [14, 15], muscle [16], fat [17], and tendon or cartilage [18, 19]. Zuk and associates [20] have successfully isolated the MSC from the adipose tissues and have been able to differentiate these to specialized cells in the presence of lineage-specific induction factors. The main purpose of these studies was to induce differentiation of precursor cells into specialized differentiated cell types.

We sought to isolate the mesenchymal stem cells from the fatty tissue and directed them to differentiate

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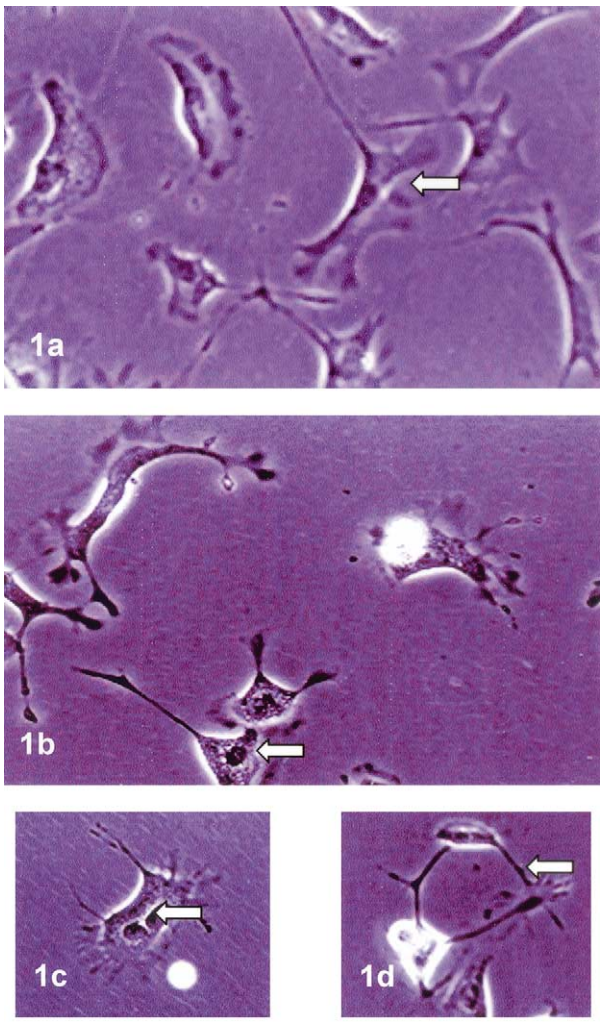

Fig 1. (a) Photomicrograph showing the adult mesenchymal stem cells that were not treated with 5-azacytidine, which served as a control (arrow) (×20). (b) Multinucleation (arrow) at 1 week after treatment with 5-azacytidine (×20). (c) Multinucleation that is characteristic of cardiomyocytes (arrow) (×20). (d) At 1 week, the treated mesenchymal stem cells extended their cytoplasmic process (arrow) with adjacent cells subsequently assuming a ball-like appearance (×20).

into cardiomyocytes. We hoped to establish a technique for cardiomyocyte transformation for use in myocyte transplantation for heart failure. Our purpose was to isolate the mesenchymal cells from abdominal subcutaneous fatty tissue culture and transform them into cardiomyocytes.

Material and Methods

Primary Culture of Rabbit Mesenchymal Stem Cells

About 100 mg of fatty tissue was harvested from the abdominal subcutaneous tissue of 10-week-old New Zealand White (NZW) rabbits, under general anesthesia. Rabbits were anesthetized with 0.3 mg/kg of hypnorm and 0.2 mg/kg of valium. Preoperatively, 0.3 mg/kg of penicillin was injected intramuscularly. Fatty tissue was

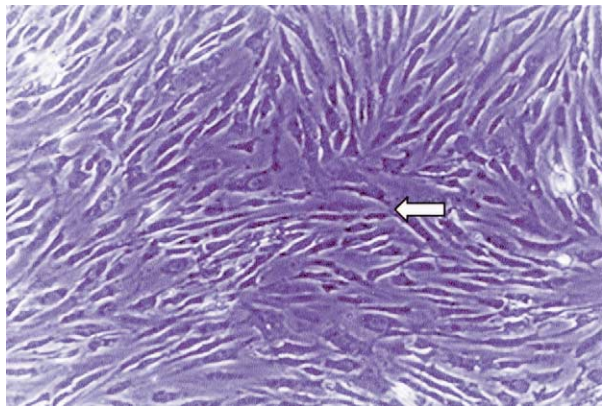

Fig 2. Isolated adult mesenchymal stem cells showing (arrow) a fibroblast-like morphology at passage 2 at 48 hours after plating (×20) (control).

washed in 0.9% (wt/vol) sodium chloride containing 200 units/mL penicillin and 200 μg/mL streptomycin. The fat was minced and digested with trypsin and collagenase supplemented with albumin for 2.5 hours in a 37°C water bath with a magnetic stir bar. The cells were centrifuged at 1,500 *g* for 10 minutes. The cell pellet was washed and resuspended in RPMI containing 15% fetal calf serum, Hepes buffer, and 1% glutamine. This was seeded into 25-cm^2 flasks and incubated at 37°C in a 95% air and 5% CO_2 incubator. The second-passaged mesenchymal cells were trypsinized with 0.25% trypsin with EDTA. The cells were centrifuged at 500 *g* for 5 minutes. The cells were then counted with a hemocytometer and plated into 25-cm^2 flasks and cultured. At 70% confluency, the mesenchymal cells were split.

Treatment With 5-Azacytidine

The twice-passaged MSCs were seeded in six-well plates at of 500, 5,000, and 50,000 cells/well. Twenty-four hours after seeding, the cells were washed with phosphate-buffered saline (PBS) twice. Then, the cells were treated with 5-azacytidine at 1, 3, 6, and 9 μmol/L and incubated

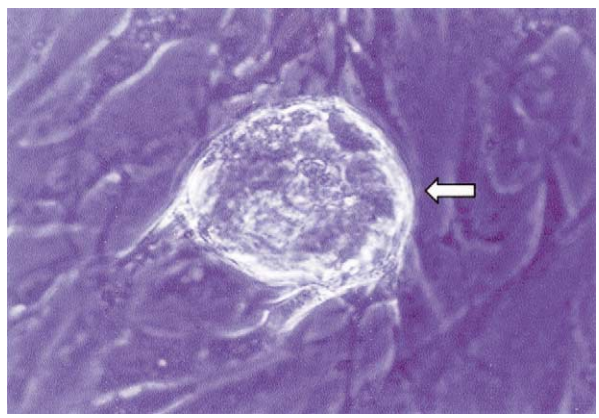

Fig 3. At 9 μmol/L and after 24 hours of incubation with 5-azacytidine, these mesenchymal stem cells began to assume a ball-like appearance at 2 weeks, as shown by the arrow *(×20).*

Fig 4. These transformed mesenchymal stem cells began to beat spontaneously at 3 weeks after treatment with 5-azacytidine, as shown by the arrow *(×20).*

for 12, 24, 48, and 72 hours, respectively. After the respective incubation periods, the cells were washed and replaced with fresh RPMI medium and incubated in a CO_2 incubator. The cells were observed daily, and the medium was changed once every in 3 days until the experiment was terminated at 2 months.

Immunostaining

The cells were fixed with 0.4% glutaraldehyde for 15 minutes at 4°C. The cells were blocked with a blocking reagent of 0.3% H_2O_2 for 30 minutes. The cells were washed three times for 5 minutes with PBS. Then, a sheep serum at 1:30 dilution was added at room temperature for 30 minutes. The serum was later drained off with blotting paper. Primary monoclonal antibody against the cardiac-specific myosin heavy chain (Chemicon Inc.) was added and incubated overnight at 4°C. On the following day, the cells were washed with PBS three times. Sufficient biotinylated link antibody was added and incubated for 30 minutes (biotinylated anti–mouse IgG, in buffered saline, containing 0.1% sodium azide). This was followed by adding streptavidin peroxide reagent (streptavidin conjugated to horseradish peroxide in buffered saline) for another 30 minutes. Later, the cells were washed and DAB was added to cover the specimen, which was incubated for 10 minutes, rinsed with distilled water, and examined under a light microscope. A similar protocol was followed for staining α-actinin with a dilution of 1:100 (Chemicon Inc.) and cardiac-specfic Troponin I (dilution 1:200) (Chemicon Inc.), respectively.

Oil Red O was used for staining adipocytes against these transformed cells. The transformed cells were washed with 0.9% sodium chloride and stained for 10 minutes with Oil Red O. The specimens were observed immediately under light microscope for lipid droplets.

Table 1. Transformation of the Adult Mesenchymal Stem Cells After 24-Hours Incubation Times Treated With 9 μmol/L of 5-Azacytidine in the Four Experiments

Experiment (24 hours)	Transformation (%)
1	23 ± 5.70
2	20 ± 7.90
3	21 ± 6.51
4	30 ± 6.12

The transformation ranged from 20% to 30%, with a mean of 23.5 ± 4.5 SD.

Table 2. Cells Seeded With 50,000 Cells and Treated With Serial Dosage of 5-Azacytidine From 1 to 12 μmol/L Over a Period of Incubation Times Ranging From 12 to 72 Hours

Incubation Time (Hours)	Dosage of 5-Azacytidine				
	1 μmol/L	3 μmol/L	6 μmol/L	9 μmol/L	12 μmol/L
12	NT	NT	NT	NT	NT
24	NT	NT	4 ± 1.5	23.5 ± 4.5	NT
48	NT	NT	NT	NT	NT
72	NT	NT	NT	NT	NT

At 50,000 cells after 24 hours of incubation at 9 μmol/L of 5-AZA, the transformation was observed. No transformation of cells was observed at 1, 3, 6, and 12 μmol/L, even with different incubation times.

NT = no transformation observed.

Results

Cultures of the fat-derived MSC assumed a fibroblast-like morphology when observed under a light microscope (Figs 1a and 2). The phenotype was maintained through repeated subcultures under nonstimulating conditions. At 1 week, after treatment with 5-azacytidine, the cells began to change their morphology (Fig 1b). The cells initially showed a remarkably slow rate of spontaneous transformation and grew with fibroblast-like morphology. Later, they showed multinucleation and extended their cytoplasmic processes with adjacent cells (Fig 1c, 1d). The cells not treated with the drug maintained their fibroblast-like morphology (Fig 2). Later, at 2 weeks, the cells aggregated and formed a ball-like appearance (Fig 3). This transformation was observed in 20% to 30% of the cells. Finally, at 3 weeks, the aggregate of cells began to

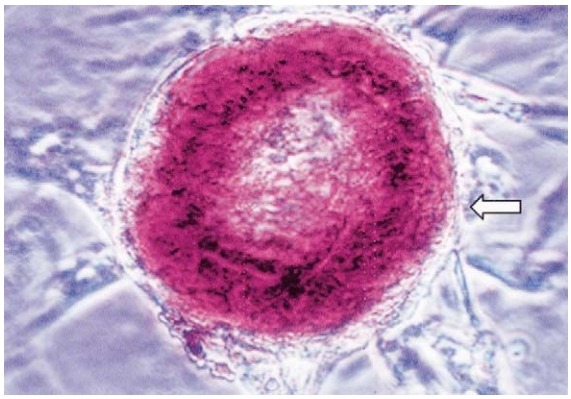

Fig 5. Light microscopy of the transformed adult mesenchymal stem cells, which assumed a ball-like appearance at 2 weeks, expressing the myosin heavy chain (arrow) *(day 40 after treating with 5-azacytidine) (×40 by immunostaining).*

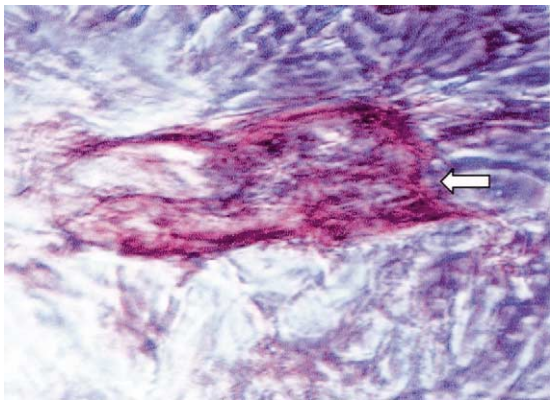

*Fig 6. Transformed stem cell expressing the α actinin (*arrow*) at 40 days after treating the mesenchymal stem cells with 5-azacytidine (×40).*

beat spontaneously (Fig 4). The cells proliferated to some extent in culture after transformation. This transformation of the stem cells was observed at 50,000 cells/well at 9 μmol/L and after incubating for 24 hours. With 50,000 cells/well, the above dose and incubation time was repeated three times, and similar phenotype transformation was observed with 23.5 ± 4.5 SD (Table 1). This showed that the results were reproducible, but the percentage of cells that transformed varied. The transformed cells did not dedifferentiate and retained their phenotype until the experiment was terminated. Other concentrations of 1, 3, 6, and 12 μmol/L of the drug or increasing the incubation time for more than 24 hours resulted in decreased proliferation of the MSC and absence of phenotypic transformation (Table 2). Immunostaining against myosin heavy chain, α actinin and Troponin-I was strongly positive (Figs 5–7). The control cells maintained their fibroblast-like morphology and proliferated at a much higher rate than those treated with 5-azacytidine (Fig 8). The cultures exposed to 5-azacytidine were observed up to 2 months after treatment. Staining against Oil Red O was negative.

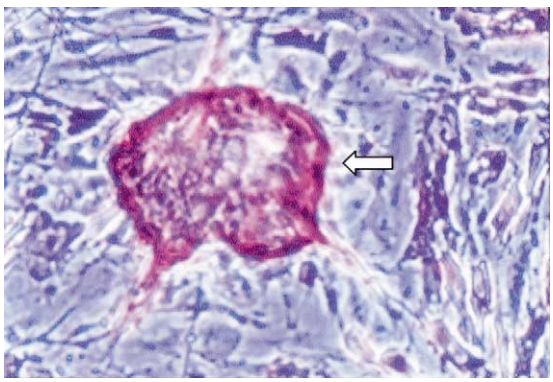

*Fig 7. Transformed stem cells expressing troponin-I at 40 days after treating with 5-azacytidine (*arrow*), which is specific for cardiomyocytes. These transformed cells began to beat spontaneously at 3 weeks (×40).*

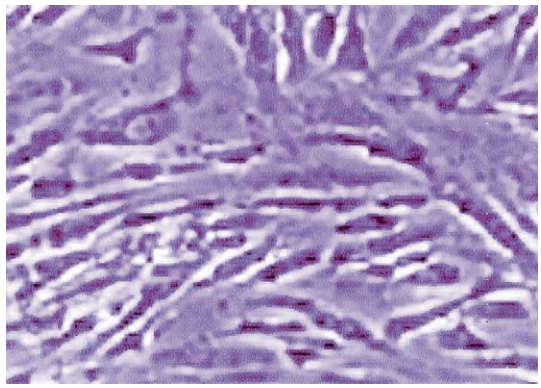

Fig 8. Light microscopy of the mesenchymal stem cells stained against myosin heavy chain shows no expression of myosin heavy chain, and no morphological changes were observed.

Comment

We have shown that fat-derived MSCs have the capacity to differentiate into cardiomyocytes after exposure to 5-azacytidine. The ideal dose was 9 μmol/L and ideal incubation time was 24 hours. The observed transformed cells exhibited the characteristic multinucleated morphology, contracted spontaneously, and stained positive against myosin heavy chain, α actinin, and Troponin-I. In our study, only 20% to 30% of the cells transformed their phenotype. The reason for this low rate of transformation could be because the MSC preparation is composed of a nonhomogenous population of cells consisting of only few true pluripotent stem cells that have the capacity to transform into cardiomyocytes. It has been shown that 5-azacytidine initiates the expression of new developmental pathways in cultured cells in the early S phase of the cell cycle [21]. In our experiment, we did not quantify the high number of cells in the S phase. This could be another reason for only 20% to 30% transformation into cardiomyocytes.

Although the striations were not observed in the culture, we observed multinucleation in the majority of the cells that formed a foci and began to beat spontaneously at 3 weeks in contrast to 7 days of C3H 10T1/2 C18 cells, which is a clonal cell line of mouse embryo cells [22]. Further, the low frequency of phenotypic conversion seen in adult rabbit cells as compared with embryonic lines might reflect differences due the age of the animals. However, these differences noted could also be due to differences in the efficiency of metabolism of 5-azacytidine and its incorporation into DNA.

Benedict and associates [23] previously reported the oncogenic transformation of 10T1/2 cells by 5-azacytidine. Although these MSCs were treated with azacytidine, oncogenic transformation was not observed in our study. The morphology of the multinucleated cells was similar to that of rat MSCs treated with 5-azacytidne, but in our study, we were not able to observe the stick-like morphology seen. Also, in our study, the cells stopped beating spontaneously at 1 week, compared with 8 weeks in Makino and associates study [24]. We were also not able to observe striations observed with the C3H 10T1/2 C18 embryonic clonal cell line [21].

In 10T1/2 and 3T3 cell lines, it was found that treatment with 5-azacytidine resulted in formation of adipocytes [25]. They found that azacytidine-induced conversion to adipocyte was concentration dependent, whereas in our study, we did not morphologically and histologically observe any adipocyte transformation.

We propose that the mesenchymal stem cells, upon demethylation with 5-azacytidine, undergo a commitment to differentiate in cardiomyocytes. First, these stem cells may enter a transient state of rapid proliferation. Upon exhaustion of their proliferative potential, these transiently amplifying stem cells may withdraw from the cell cycle and become terminally differentiated into cardiomyocytes, subsequently switching off the telomerase gene.

The use of MSCs to regenerate skeletal tissue [26, 27] in clinical situations is well known. But for the first time, our study showed that the adult MSCs from the subcutaneous fat could be transformed in to cardiomyocytes. As seen in this study, it may be possible to pretreat autologous MSCs with 5-azacytidine before transplantation into infarcted myocardium to insure that the MSCs will efficiently and rapidly regenerate the injured myocardium. The clinical use of culture-expanded MSCs may be to repair massive myocardial defects that are too extensive to be treated otherwise. The use of agents like 5-azacytidine may be to enhance such MSC-mediated cardiomyocyte regeneration. These transformed cells should ideally be transplanted 1 week after transformation into the infracted myocardium, to avoid dedifferentiation in the culture, because cardiomyocytes are known to redifferentiate after prolonged culture [28]. The use of MSCs would be advantageous compared with other sources of stem cells for cell transplantation because acquisition of fat from the abdomen is a rapid, routine outpatient procedure.

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