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ORIGINAL CONTRIBUTION

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ABSTRACT

There is increasing interest in developing cell-based therapies to regenerate functional muscle and blood vessels in infarcted dysfunctional myocardium, using stem cells resident in the adult heart. The objective of our study was to identify an easy and cost-effective method for the isolation and expansion of human adult cardiac-resident stem cells. The cells were isolated from right atrial biopsy samples obtained from patients with ischemic heart disease, who were undergoing coronary artery bypass grafting. Two different isolation methods, enzymatic and nonenzymatic, were employed. The cell yield and cluster formation were not significantly different with either of the techniques used for cell isolation. The nonenzymatic method is recommended because of its simplicity and lower cost compared to the enzymatic method.

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INTRODUCTION

Myocardial regeneration has gained much attention in recent years. Evidence provided by Beltrami and colleagues[1] of myocyte replication in postnatal human hearts following ischemic injury suggested the existence of a resident pool of stem cells in the heart. Morphologically, these cells are small and round with a large nucleus surrounded by a thin rim of cytoplasm.[2] They have been found to proliferate, attain the morphological and functional characteristics of cardiac myocytes, and contract synchronously with the existing cells of the heart.[3] They can be successfully isolated from a small biopsy specimen, and animal experiments suggest their clinical utility in regenerating the failing heart.[4] The number of stem cells that can be obtained from biopsy tissue is relatively small, and they have to be cloned to acquire sufficient amounts for therapeutic use. The methods adopted for isolation of stem cells have to be selected so that maximum cell yield and viability are ensured. We obtained right atrial biopsies with the objective of identifying a suitable method for isolation and cloning of resident cardiac stem cells. The results of 2 different isolation procedures, enzymatic and nonenzymatic, are reported here.

PATIENTS AND METHODS

Right atrial biopsy samples were obtained from 10 patients, 2 women and 8 men, aged 42 to 72 years, with ischemic heart disease, who underwent coronary artery bypass grafting at our institute. The samples were taken from the site of right atrial cannulation. The study was carried out with the approval of the institutional ethics committee, and informed consent was obtained from those whose tissues were collected for the study.

In the enzymatic procedure, cardiac stem cells were isolated from freshly collected atrial biopsies as described by Messina and colleagues.[5] Briefly, the isolated myocardial tissue was minced into 1–3-mm^3 pieces and washed with Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS) several times to remove fat and contaminating red blood cells. The tissue fragments

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were digested 3 times for 5 min at 37°C with 0.2% trypsin (Sigma-Aldrich, St Louis, Missouri, USA) and 0.1% collagenase IV (Invitrogen, Grand Island, New York, USA). Cells obtained from the initial digests were discarded, and the remaining tissue fragments were washed with Iscove's Modified Dulbecco's Medium (IMDM; Sigma-Aldrich) supplemented with 10% fetal calf serum (Sigma-Aldrich), 0.1 mmol·L^{-1} 2-mercaptoethanol (Sigma-Aldrich), 2 mmol·L^{-1} L-glutamine (Sigma-Aldrich), 100 U·mL^{-1} benzyl penicillin (Hindustan Antibiotics Ltd., Pimpri, Pune, India), and 100 g·mL^{-1} streptomycin (Hindustan Antibiotics Ltd.). The cells were seeded on gelatin-coated culture dishes and incubated at 37°C in 95% air + 5% CO_2. In the nonenzymatic procedure, after sufficient washing with Ca^{2+}- Mg^{2+}-free PBS, the sample tissue was minced into 1–2-mm^3 pieces and cultured as explants in gelatin-coated culture dishes in the above-mentioned culture medium. Every 2 days, the medium was changed without disturbing the loosely adherent explants.

The migrated cells from both procedures were characterized for the presence of the stem cell marker, ckit, by immunocytochemical staining and magnetic-activated cell sorting (MACS; Miltenyi Biotech, Bergisch Gladbach, Germany). Cells were washed with PBS sufficiently to remove the medium, and stained with a monoclonal antibody to ckit (Santa Cruz Biotechnology, CA, USA), the receptor for stem cell factor. Fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin G (Molecular Probes, Leiden, The Netherlands) was used as the secondary antibody. Samples were processed for fluorescence microscopy. Cells were also stained with a monoclonal antibody to von Willebrand Factor (Sigma-Aldrich) and vimentin (Dako North America, Inc., Carpinteria, CA, USA). Samples were processed using a Dako Cytomation LSAB + System-HRP kit (Dako North America, Inc.), and counterstained with hematoxylin, which stains the nucleus blue.

Characterization of the isolated small round cells for ckit expression was performed by MACS using an anti-human CD117 micro-bead kit, according to the manufacturer's manual. Briefly, the migrated phase-bright cells were collected by trypsin-EDTA treatment, pooled and incubated with monoclonal anti-human ckit antibody-coated micro-beads. The cell suspension was passed through a magnetic column placed in a strong magnetic field. The unlabeled cells (ckit$^-$) were washed out using MACS buffer, and the labeled (ckit$^+$) cells were retained in the MACS column. The column was separated from the magnetic field, and the ckit$^+$-cell fraction was collected. The ckit$^+$ cells sorted by MACS were stained with Hoechst 33258, which stains the nucleus blue, and examined under a fluorescent microscope.

Cells that migrated from explants of both procedures were similarly processed. They were collected by pooling 2 washes with Ca^{2+}- Mg^{2+}-free PBS, one wash with EDTA, and then with trypsin/EDTA (0.05% trypsin + 0.02% EDTA) at 37°C. After passing through a 50-micron nylon mesh, the collected cells were re-suspended in a growth medium of IMDM + Dulbecco's Modified Eagle's Media-Ham's F-12 mix (Sigma-Aldrich) supplemented with 0.1 mmol·L^{-1} 2-mercaptoethanol, 10 ng·mL^{-1} epidermal growth factor (US Biologicals, Swampscott, Massachusetts, USA), 20 ng·mL^{-1} basic fibroblast growth factor (US Biologicals), 2% B27 serum supplement (Invitrogen), 40 nmol·L^{-1} thrombin (Sigma-Aldrich), 100 U·mL^{-1} benzyl penicillin, 100 g·mL^{-1} streptomycin, 2 mmol·L^{-1} L-glutamine.[5] The cells were seeded in poly-D-lysine-coated 4-well culture dishes (BD Biosciences, San Jose, CA, USA) at a density of 30,000 cells per well. Within 24 hours of culture, the seeded cells formed loosely adherent small round clusters (cardiospheres). The cardiosphere cells were quantitatively characterized for the expression of stem cell markers, ckit, multiple drug resistance-1 (MDR-1), and CD34, and endothelial cell markers by fluorescence-activated cell-sorting analysis. Different culture substrates, such as gelatin (Sigma-Aldrich), fibronectin (BD Biosciences), and poly-D-lysine (BD Biosciences), were employed to quantify cardiosphere yield. A minimum of 50 randomly selected fields were counted in each culture dish, and the number of cardiospheres per cm^2 was calculated. Cardiosphere formations under different growth conditions, such as IMDM/poly-D-lysine dish, IMDM/uncoated dish, growth factor-enriched medium/uncoated dish, and growth factor-enriched medium/poly-D-lysine dish were also analyzed. The cardiospheres responded to electrical stimulation: contraction was observed when the cardiospheres were stimulated using bipolar constant-current pulses delivered via platinum electrodes placed 1 cm apart.

For characterization of cardiosphere cells by fluorescence-activated cell-sorting analysis, the cardiospheres were first washed with PBS to remove the growth media completely, followed by dissociation with trypsin-EDTA (1×) solution for 1–2 min at 37°C. Undissociated clusters were subjected to mechanical trituration. The cells were collected and centrifuged at 2,000 rpm for 5 min. After sufficient PBS washes, the cells were incubated with primary antibodies (Santa Cruz Biotechnology) against CD117, MDR-1, CD34, von Willebrand factor, cardiac troponin I and myosin heavy chain markers at 4°C for 30 min. After incubation, the cells were washed with cold PBS to remove unbound primary antibodies, then incubated with fluorescent-labeled secondary antibodies (Molecular Probes) for 30 min at 4°C. Samples were washed with

Table 1. **Characteristics of 10 Patients with Ischemic Heart Disease**

Variables	No. of Patients
Age (years)	58 ± 10
Male sex	8
Hypertension	5
Smoker	7
Diabetes mellitus	6
Dyslipidemia	5
1-vessel disease	0
2-vessel disease	5
3-vessel disease	5
NYHA functional class	
I	0
II	8
III	2
IV	0

NYHA = New York Heart Association.

cold PBS to remove unbound antibodies, fixed with 4% paraformaldehyde, and stored at 4°C until analysis. The samples were analyzed with a 4-color multiparameter flow cytometer (FACS Aria; Becton Dickinson, San Jose, CA, USA).

Data are expressed as mean ± standard deviation. Comparisons between groups were analyzed by the 2-tailed Student's *t* test.

RESULTS

The clinical characteristics of the 10 patients from whom biopsies were taken are summarized in Table 1. With both isolation methods, after a period of incubation ranging from 1–3 weeks, a layer of fibroblasts was generated from well-adherent explants, over which small round cells were seen to be migrating. The majority of the phase-bright cells were present in the vicinity of the explants (Figure 1A and 1B). The maximum number of small round phase-bright cells was observed during the 3rd week of explant culture with both methods. Migrated cells stained positive for ckit, and negative for vimentin and von Willebrand factor expression. Since migrated cells were fewer in number and not well adherent to the culture surface, the number of positively stained cells per field was small. MACS sorted ckit$^+$ cells were stained with Hoescht 33258. Approximately 20% enrichment was obtained after MACS procedures: the cell count was 125,000 cells·mL^{-1} before MACS and 25,000 cells·mL^{-1} after MACS.

The ckit-positive cells isolated by both methods were able to form clusters in a growth factor supplemented

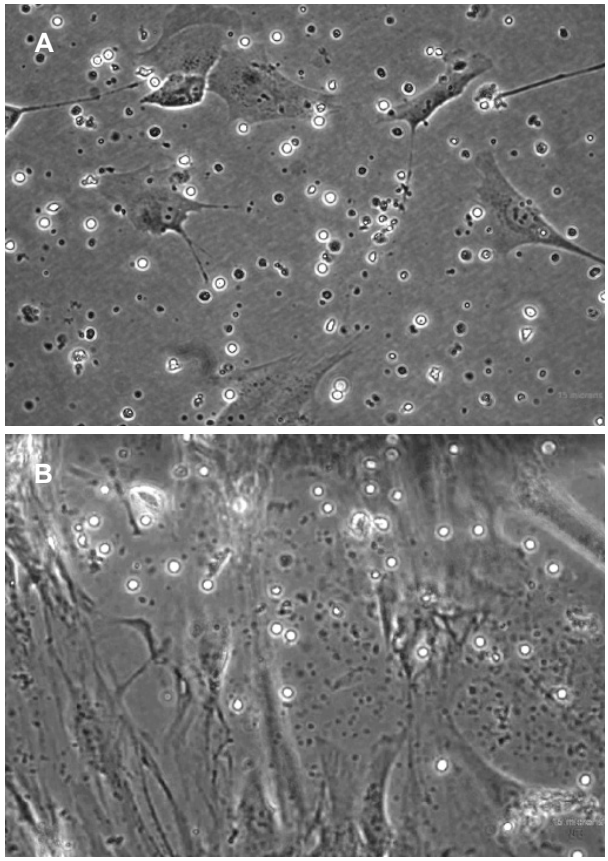

Figure 1. Phase-contrast micrograph of migrating phase-bright cells over fibroblast-like cells; (**A**) Day 7, nonenzymatic method, (**B**) Day 7, enzymatic method. Scale bar = 15 microns.

medium (Figure 2A and 2B). The size of the cardiospheres varied from 20 to 172 microns. Within 2–3 days of culture, the cardiospheres increased in size, and some detached from the culture surface (Figure 2C). Floating cardiospheres were mechanically dispersed and re-seeded. Some were firmly attached even on the 28th day of culture (Figure 2D). Firmly attached fibroblast cells formed a network pattern in the poly-D-lysine culture dish. Among the different growth conditions and culture substrates (gelatin, fibronectin, and poly-D-lysine) employed, cardiosphere formation was observed only in cells seeded in poly-D-lysine-coated dishes supplemented with growth factors. With other substrates, only fibroblasts were confluent; cardiosphere formation was not observed. The initial count of the migrated phase-bright cells varied significantly from patient to patient. There were instances of high cardiosphere count when there was a very low primary yield. The results of a comparative analysis between enzymatic and nonenzymatic isolation methods for the initial cell count and number of cardiospheres formed after culture enrichment are given in Table 2.

After expansion in a medium supplemented with growth factors, cardiosphere-forming cells were positive for the expression of ckit and MDR-1 (Figure 3). On the 30th day

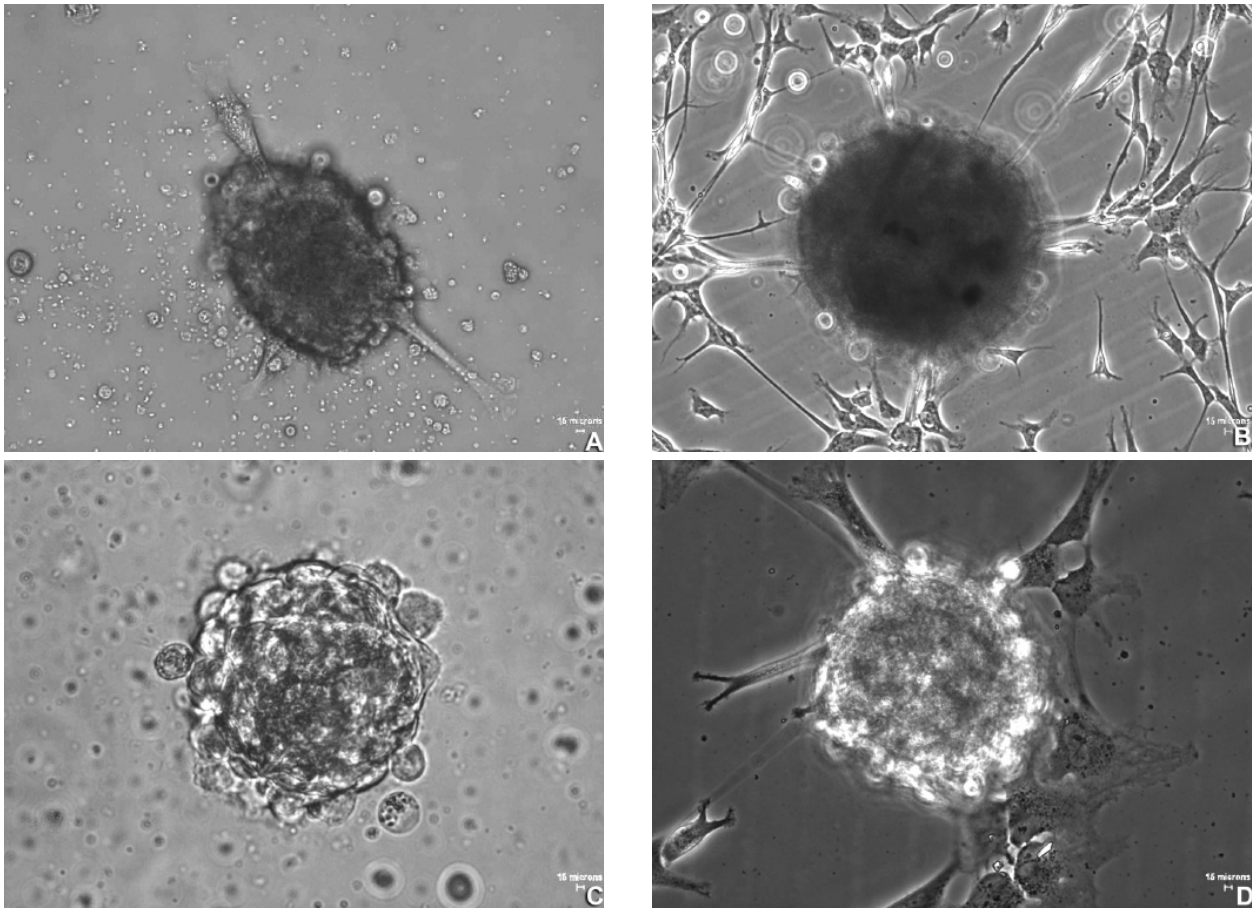

Figure 2. Adherent cardiospheres on a poly-D-lysine-coated dish; (**A**) Cardiospheres obtained by the enzymatic isolation method, (**B**) Cardiospheres obtained by the nonenzymatic isolation method, (**C**) A floating cardiosphere after 72 hours of culture, (**D**) A well-adherent cardiosphere on the 28th day of culture. Scale bar = 15 microns.

Table 2. **Comparison of Nonenzymatic and Enzymatic Stem Cell Isolation Procedures**

Nonenzymatic		Enzymatic	
Initial Cell Count ($cells \cdot mL^{-1} \cdot g^{-1} \times 10^5$)	Cluster Count ($clusters \cdot cm^{-2} \times 10^2$)	Initial Cell Count ($cells \cdot mL^{-1} \cdot g^{-1} \times 10^5$)	Cluster Count ($clusters \cdot cm^{-2} \times 10^2$)
6.46	206	7.91	321
4.59	580	5.00	407
2.66	489	5.09	389
5.08	257	3.33	367
3.45	531	2.47	310
Mean 4.45 ± 1.5	Mean 4.12 ± 169	Mean 4.76 ± 2.08*	Mean 3.59 ± 42†

*Initial counts nonenzymatic vs enzymatic, $p > 0.5$. †Cluster count nonenzymatic vs enzymatic, $p > 0.5$.

of cardiosphere culture, 84% of cells simultaneously expressed markers for ckit and MDR-1 (Figure 3B), and 94% of cells were positive for CD34 expression (Figure 3C). The cells stained positively for expression of the endothelial marker, von Willebrand factor (Figure 3D), and for the cardiac differentiation markers, myosin heavy chain and troponin I. Simultaneous expression of cardiac troponin I and myosin heavy chain was shown by 82% of cardiosphere cells on the 30th day of culture (Figure 3E). Fluorescence-activated cell-sorting analysis of primary cells derived from the explants showed 31% positivity for CD117 marker expression; the results for the cardiosphere-derived cells obtained by both methods are shown in Figure 4.

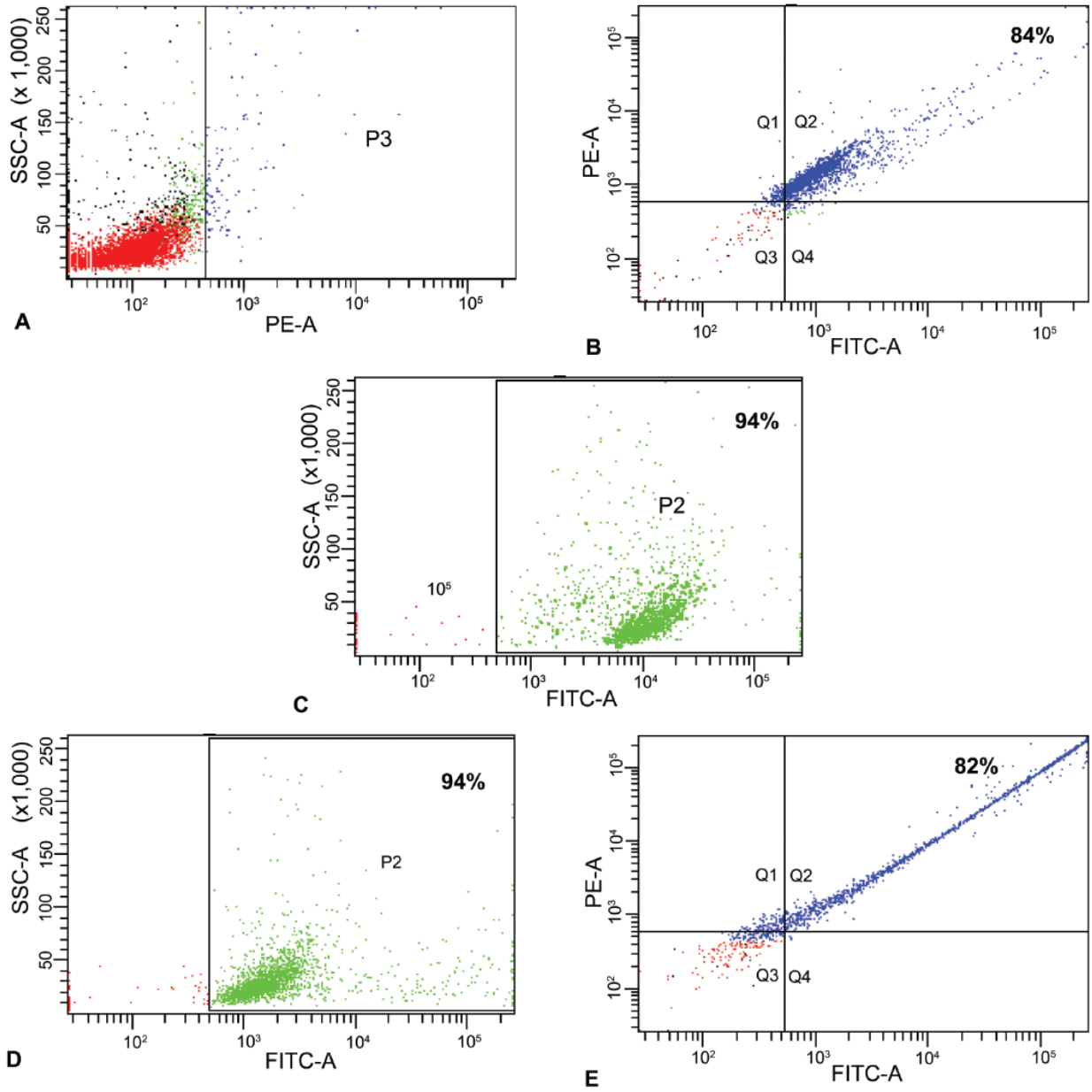

Figure 3. Fluorescence-activated cell-sorting analysis of cardiosphere-forming cells; (**A**) Control. (**B**) Cardiosphere cells showing 84% positivity for both ckit and multiple drug resistance-1 expression, (**C**) Phenotype profile for CD34, (**D**) von Willebrand factor expression shown as a percentage of positive events, (**E**) Cardiosphere cells showing 82% positivity for both cardiac troponin I and myosin heavy chain expression. SSC = side scatter (complexity/granularity of sorted cells), PE = phycoerythrin, FITC = fluorescein isothiocyanate, Q1 = quadrant with cells positive for PE-labeled antibody, Q2 = quadrant with cells positive for both PE- and FITC-labeled antibodies, Q3 = negative cell populations, Q4 = cells positive for FITC-labeled antibody. P2 = positive cells when a single antibody is used for staining.

Discussion

Our results suggest that ckit$^+$ cells can be isolated and grown into cardiospheres by simple explant culture methods. The cell yield and cardiosphere count were comparable with those of the enzymatic method. The time lag in sample preparation is less, and costly enzymes (collagenase and trypsin) are not required in the nonenzymatic method. As the digestion step is bypassed in this approach, there is less chance of tissue damage.

Following the reported possibility of a pool of adult clonogenic stem cells in postnatal human heart, which was enhanced after myocardial infarction and attenuated during chronic heart failure, these cells were successfully isolated and cloned from animal hearts.[1,4,5–7] The resident cells or their clonal progeny can reconstitute a well-differentiated myocardial wall when injected into infarcted ventricle.[8] Adult cardiac-resident stem cells thus provide an effective means of regenerating damaged

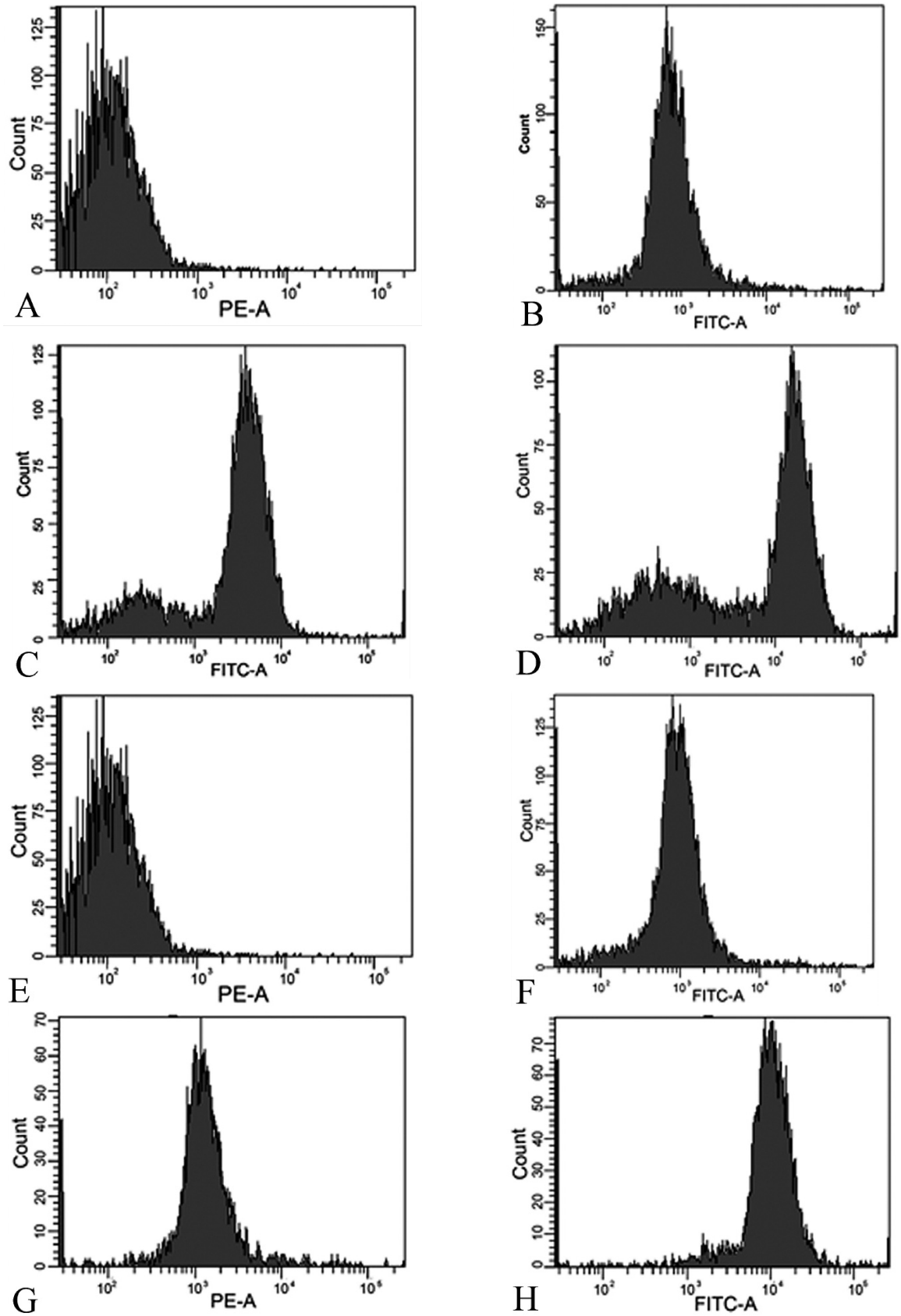

Figure 4. Fluorescence-activated cell-sorting analysis of cardiosphere-forming cells obtained by the enzymatic and nonenzymatic methods; **A**, **B**, **C**, **D** = control, ckit, multiple drug resistance-1, and CD34 expression, respectively, for the enzymatic method, **E**, **F**, **G**, **H** = control, ckit, multiple drug resistance-1, and CD34 expression, respectively, for the nonenzymatic method.

myocardium. Compared to other cell types (such as bone marrow-derived cells), they are believed to be faster in achieving the structural and functional characteristics favorable for regenerating damaged myocardium. To our knowledge, there are only 2 previous reports of successful isolation of human adult cardiac stem cells.

Stem cell niches are reported to provide a microenvironment necessary for preserving the survival and replication potential of stem cells.[9] Cells sheltered in niches have a much higher potential for self-renewal than primitive cells that divide rarely.[10] This explains the increase in stem cell number when cultured in a growth factor-enriched medium that might mimic the conditions prevailing in a stem cell niche in the heart. The cells migrated from explants grown without enriched medium were comparatively few in number. Adult cardiac stem cells undergo lineage commitment, and they divide and differentiate into myocytes, smooth muscle cells, and endothelial cells, under the appropriate stimulus.[11,12] The migrated cells stained negative for vimentin and von Willebrand factor expression, suggesting that they have not yet entered into endothelial/fibroblast lineages. The cells retained their undifferentiated nature, confirmed by ckit marker expression. When the cells were grown in a growth factor-enriched medium, they formed clusters of dividing cells (cardiospheres). Cardiospheres consist of a mixture of cardiac stem cells and differentiating progenitors.[5] The cardiosphere cells stained positive for the endothelial marker, von Willebrand factor, and also for the cardiac differentiation markers, myosin heavy chain and cardiac troponin I, demonstrating the ability to differentiate into distinctive lineages under proper stimulus.

Our study did not address the possible effects of factors such as the clinical and risk-factor profile of the patients in determining the cardiac stem cell yield. It is also unclear whether all the migrated ckit$^+$ cells in the primary explant culture would retain their inherent property of stem cells when grown for a long period in culture conditions to obtain sufficient cells for clinical use. However, our study confirms that ckit/MDR-positive cardiosphere-forming cells can be isolated from small biopsy samples from patients with ischemic heart disease, employing nonenzymatic methods. On continued culture, these cells exhibited characteristic cardiac differentiation markers, such as cardiac troponin I and myosin heavy chain, which compels us to suggest that they are indeed stem cells resident in the heart.

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