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Cellularity and structure of fresh human coronary thrombectomy specimens; presence of cells with markers of progenitor cells

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Abstract

Acute coronary syndromes and acute myocardial infarctions are often related to plaque rupture and the formation of thrombi at the site of the rupture. We examined fresh coronary thrombectomy specimens from patients with acute coronary syndromes and assessed their structure and cellularity. The thrombectomy specimens consisted of platelets, erythrocytes and inflammatory cells. Several specimens contained multiple cholesterol crystals. Culture of thrombectomy specimens yielded cells growing in various patterns depending on the culture medium used. Culture in serum-free stem cell enrichment medium yielded cells with features of endothelial progenitor cells which survived in culture for a year. Immunohistochemical analysis of the thrombi revealed cells positive for CD34, cells positive for CD15 and cells positive for desmin *in situ*, whereas cultured cell from thrombi was desmin positive but pancytokeratin negative. Cells cultured in endothelial cell medium were von Willebrand factor positive. The content of coronary thrombectomy specimens is heterogeneous and consists of blood cells but also possibly cells from the vascular wall and cholesterol crystals. The culture of cells contained in the specimens yielded multiplying cells, some of which demonstrated features of haematopoietic progenitor cells and which differentiated into various cell-types.

Keywords: coronary • thrombus • acute coronary syndrome • platelets • endothelial progenitor cells • thrombectomy

Introduction

Acute coronary syndromes and acute myocardial infarctions are associated with plaque rupture and the formation of thrombi at the site of the rupture [1, 2]. Although the presence of thrombi in the coronaries of patients with acute coronary syndromes has been suggested by coronary angiography [3] and confirmed by autopsy studies [4, 5], there is little information about fresh human coronary thrombi. Newer suction catheters make the percutaneous retrieval of fresh thrombi from the coronaries of patients possible and may contribute to improved perfusion [6, 7]. A recent review by Gerd Heusch and colleagues [7] provides an overview on the previously published data on the molecular and cellular composition of particulate debris as well as soluble factors retrieved from human coronary arteries, primarily as this pertains to coronary microembolization. We examined fresh coronary thrombectomy specimens from patients with acute coronary syndromes and assessed their structure and cellularity as well as the potential of thrombi to participate in vascular healing.

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Materials and methods

The thrombectomy specimens were aspirated from patients presenting with acute coronary syndromes [ST-segment and non-ST-segment elevation myocardial infarction (MI)] to the cardiac catheterization laboratory of the Yale- New Haven Hospital. Because of concern about the process of obtaining consent from patients before their acute treatment of myocardial

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infarction and the fact that this could have delayed life-saving therapy, the study was performed with approval of the Yale University human investigation committee under a protocol that specified that consent was not needed if no patient data were collected and that at least one investigator had to have no contact with the patients or with the specimens. Thus, the thrombectomy specimens could not be linked to specific patient data.

Fresh thrombectomy specimens were retrieved with an Export (Medtronic, San Diego, CA, USA) aspiration coronary catheter from patients with acute coronary syndromes during clinically indicated coronary angiography and percutaneous coronary intervention procedures. The decision to use a coronary aspiration catheter was the clinical decision of the interventional cardiologist. The specimens were placed in the collection filter, which was then immersed in normal saline in a sterile biopsy container.

The specimens were then aspirated with a pipette under a sterile hood and placed in culture bottles with medium. DMEM high glucose medium (Invitrogen, Carlsbad, CA, USA) with Penicillin/Streptomycin and 20% FBS (Invitrogen) was used for the culture of some thrombectomy specimens and the cells grew and developed features of mesenchymal cells. M199 medium (Invitrogen) with Penicillin/Streptomycin and 20% FBS (Invitrogen), L-glutamine, endothelial cell growth supplement (BD Biosciences, Mountain View, CA, USA) and 100 μg/ml porcine intestinal heparin (Sigma, St. Louis, MO, USA) was used for the culture of other thrombectomy specimens and these yielded cells that differentiated into endothelial cell lineages [8]. Low serum stem cell medium (HPGM, Cambrex, Walkersville, MD, USA) was used to select progenitor cells. The cells were observed under the inverted microscope daily. When they reached confluence, they were detached with trypsin and passaged onto cell culture dishes and/or onto slides culture chambers and stained as described below.

Microscopy

Other specimens were fixed in paraformaldehyde, embedded in paraffin and subsequently sectioned. Sections were stained with haematoxylin–eosin or were incubated with primary anti-human desmin, pancytokeratin, cluster of differentiation (CD)31 [platelet endothelial cell adhesion molecule (PECAM-1)], TGFb (transforming growth factor beta), von Willebrand factor (vWF), CD117 [Mast/stem cell growth factor receptor (SCFR)], CD15 [stage-specific embryonic antigen 1 or Lewis X (a surface marker for myelomonocytic cells/neutrophils)] and CD34 [a glycoprotein (L-selectin receptor) found on bone marrow haematopoietic cells, endothelial progenitor cells and endothelial cells] antibodies, then with secondary biotinylated antibody and detected with a streptavidin-horseradish peroxidase system after background staining. Cells cultured in cell culture media were also subjected to immunohistochemistry with the above antibodies and also human anti-CD68 [macrosialin (a glycoprotein expressed on monocytes/macrophages)] and muscle sarcoplasma actin [(MSA) a marker for muscle cells].

To determine the relative contribution of various thrombus components, planimetry using Image J software (http://rsbweb.nih.gov/ij/) was used. Because the distinction between fibrin material and platelets is difficult, the software was used to detect and express the proportion of red cell material with the rest being non-red cell material (fibrin, platelets and white cells).

Cells grown in serum-poor stem cell medium were also plated onto 24-well plates coated with fibronectin and then incubated with DiI LDL (Sigma) to assess their ability to endocytose LDL [9].

Results

Thrombectomy specimens were retrieved from 29 patients; samples from 10 patients were used for histology, but one sample was too small and could not be analysed; samples from the other 19 patients were cultured, but only 16 grew and could be passaged. All patients were pretreated with aspirin and clopidogrel and anticoagulated with intravenous heparin to achieve and activated clotting time 250–

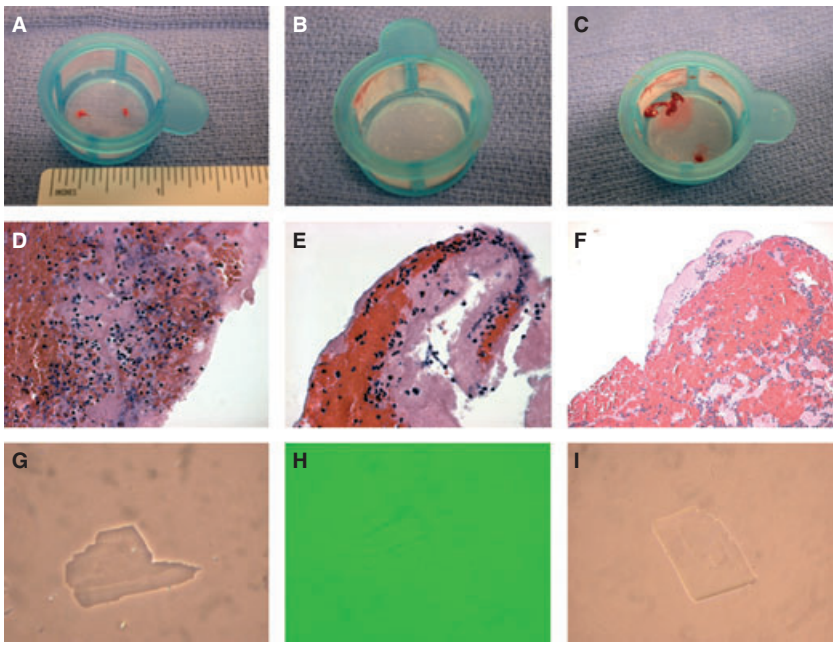

Fig. 1 Mixed thrombi, white thrombi and red thrombi (**A**–**C**); platelets, fibrin, red cells and white cells in varying amounts (**D**–**F**); cholesterol crystals of various shapes (**G**–**I**).

300 sec. as part of a standard protocol. In general, the proportion of patients with acute coronary syndrome treated with glycoprotein IIb/IIIa inhibitors was less than 20%.

Thrombectomy specimens were both white and red thrombi. They contained blood cells and fibrin in various proportions. They also contained cholesterol crystals (Fig. 1). The relative contribution of red cell material in the nine samples used for histology was 51 ± 18%.

Immunohistochemistry of thrombi *in situ* after fixation with paraformaldehyde: CD34 positive, desmin (a marker for muscle cells) positive, pancytokeratin (a marker for epithelial cells) negative, CD31 (a marker for endothelial cells) positive, CD15 (a marker for neutrophilic polymorphonuclear cells) positive, TGFb (a marker for smooth muscle cells and macrophages) positive, vWF (a marker for endothelial cells) positive, CD117 (a marker for haematopoietic stem cells) negative (Fig. 2).

Cells grown in DMEM 20% FBS medium became spindle shaped and grew in clusters until they became confluent. There were numerous dividing cells and sporadic multinucleated cells (Fig. 3A–G). Cells grown in M199 20% FBS (endothelial cells medium) grew in small clusters and then became confluent in a cobblestone pattern (Fig. 3H–I).

Cells survived in serum-free stem cell medium for 12 months without dividing (Fig. 4A). Cells grown in serum-poor stem cell med-

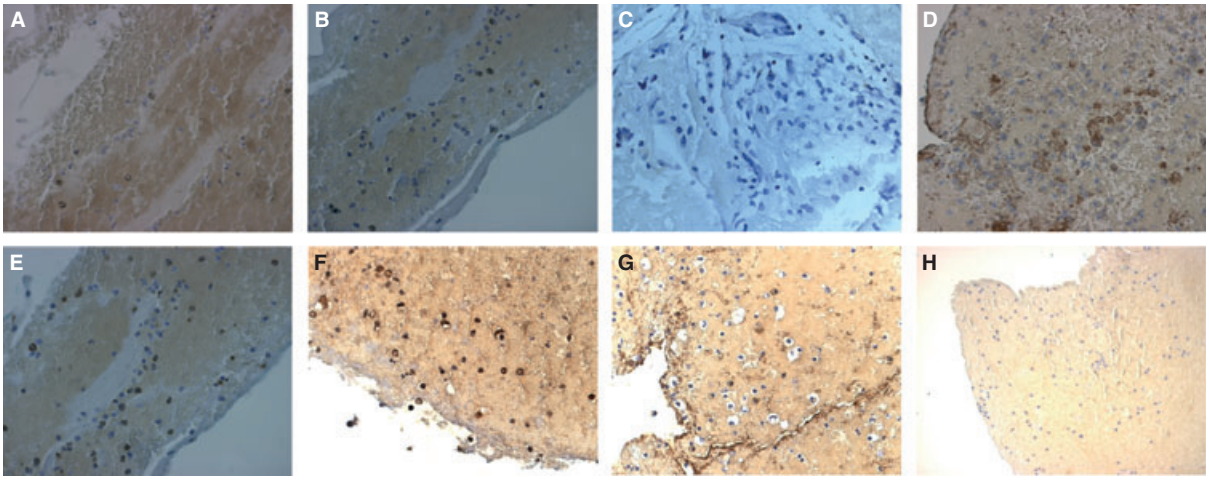

Fig. 2 Immunohistochemistry of thrombi *in situ* after fixation with paraformaldehyde: CD34, desmin, pancytokeratin (negative), CD31 (**A**–**D**), CD15, TGFb, vWF, CD117 (negative) (**E**–**H**).

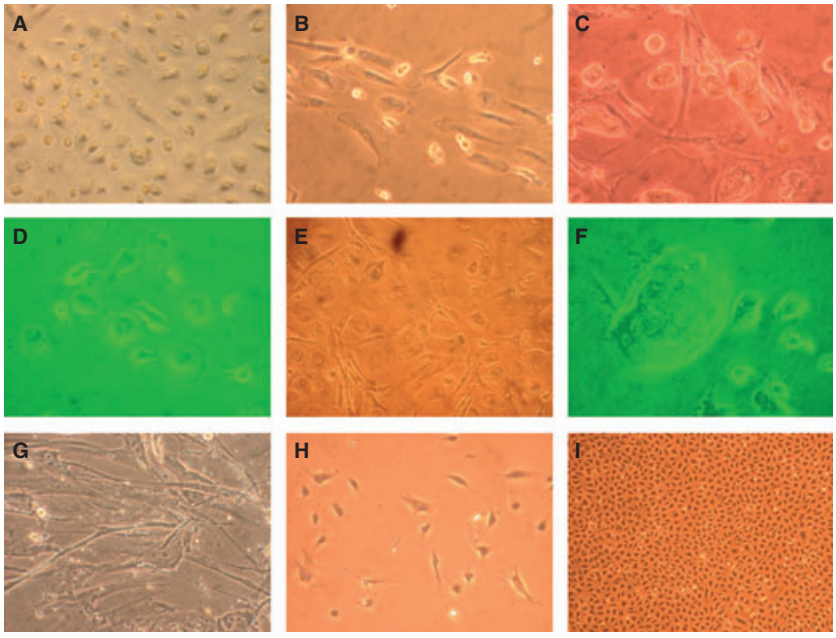

Fig. 3 Growth pattern of cultured cells grown in DMEM 20% FBS (**A**–**C**); shapes of cultured cells, dividing cells, multinucleated cells (**D**–**F**), spindle-shaped cells reminiscent of mesenchymal cells/fibroblasts (**G**); cells grown in M199 20% FBS (EC medium) grew in small clusters (**H**) and then became confluent in a cobblestone pattern (**I**).

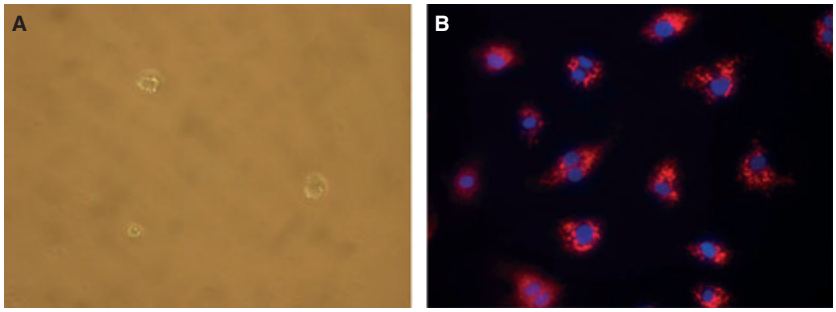

Fig. 4 Cells survived in serum-free stem cell medium for 12 months (**A**); mononuclear cells from thrombus grown in serum-poor stem cell medium differentiated to endothelial progenitor cells capable of endocytosing Dil-ac LDL (**B**).

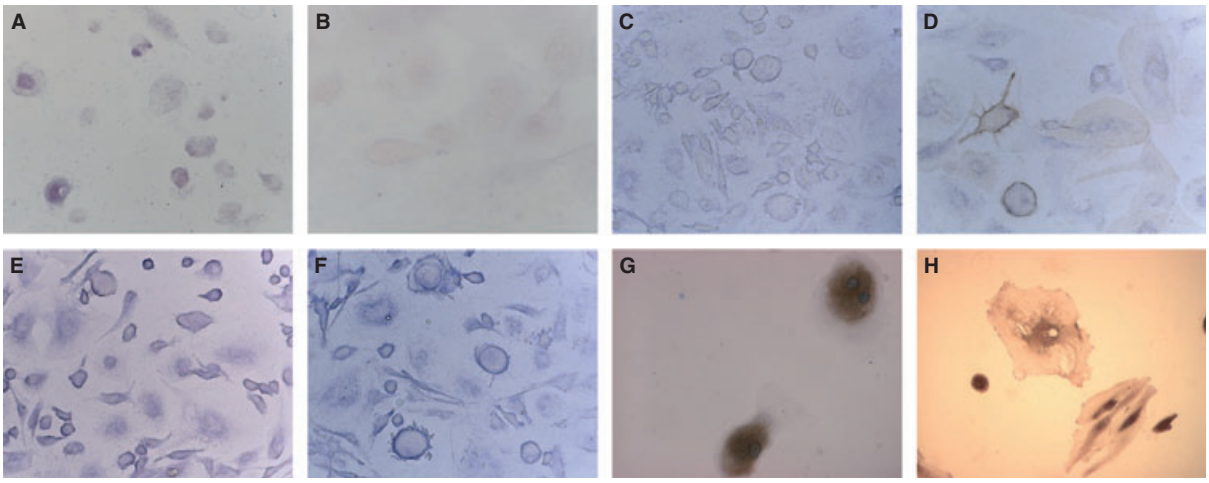

Fig. 5 Immunohistochemistry of cells grown in DMEM 20% FBS: CD68 (negative), vWF (negative), CD34 (negative), MSA (**A**–**D**), pancytokeratin (negative), CD15 (negative), desmin, TGF (**E**–**H**).

ium were then incubated with endothelial cells medium, differentiated to endothelial progenitor cells capable of endocytosing Dil-ac LDL (Fig. 4B). Cells grown in growth factor-enriched stem cell medium were passaged 12–14 times.

Cells grown in DMEM 20% FBS were CD68 (a marker for monocytes) negative, vWF negative, CD34 negative, MSA positive, pancytokeratin (a marker for epithelial cells) negative, CD15 negative, desmin positive and TGFβ positive (Fig. 5).

Cells grown in M199 20% FBS were pancytokeratin negative, CD31 (a marker for endothelial cells) positive, CD34 positive, vWF positive (Fig. 6).

Discussion

Coronary thrombi are white, red and mixed thrombi and contain various amounts of platelets, red blood cells and leucocytes [10]. This is different than the classic teaching that venous thrombi are red and arterial thrombi are white, and is in accordance with previously published data by Montalescot *et al.* who showed that the composition of coronary thrombi can change depending on the timepoint when they are harvested in relation to their formation [11, 12]. The variability in cellular content may result in changes in clot mechanical properties [13]. Leucocyte infiltration of unstable plaques has been described before [14] and may be related to outcome in patients with acute coronary syndromes. Thus, the variability in thrombus composition may be caused by both intrinsic characteristics of the patient (*e.g.* peripheral blood white cell count) and may reflect the lag between plaque rupture/coronary thrombosis and cardiac catheterization and percutaneous coronary intervention and thrombectomy. A recent report that studied the structure of coronary thrombi and its relation to the onset of chest pain, found that in at least 50% of patients with acute STEMI, coronary thrombi were days or weeks old. The authors interpreted this as suggesting that sudden coronary occlusion is often preceded by a variable period of plaque instability and thrombus formation, initiated days or weeks before onset of symptoms [15]. We also found cholesterol crystals which have been described in plaques before and which may contribute to thrombosis in ruptured plaque and to the so called 'no-reflow'[16, 17] phenomenon which is seen in the cardiac catheterization laboratory.

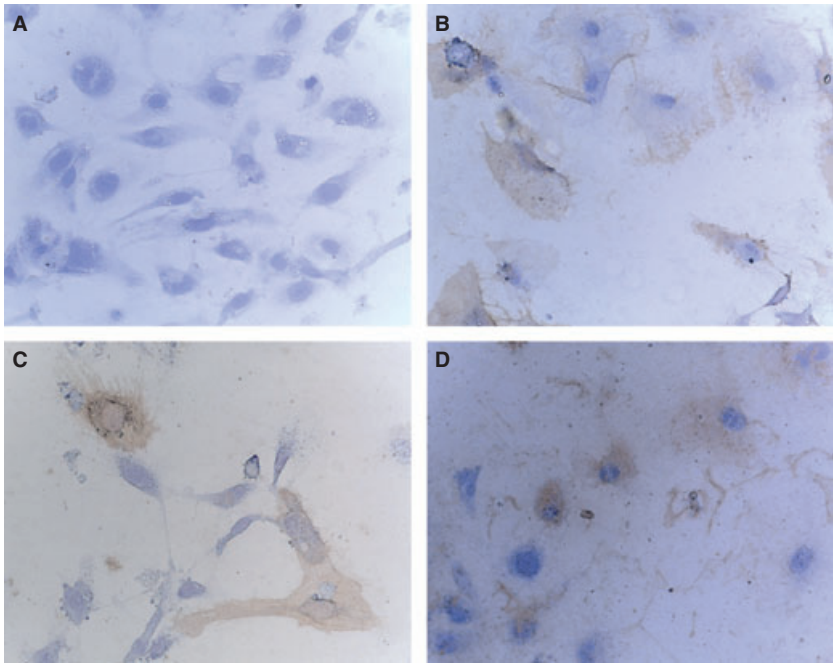

Fig. 6 Immunohistochemistry of cells grown in M199 20% FBS: pancytokeratin (negative), CD31, CD34, vWF (**A**–**D**).

Blood-derived cells are present in approximately 10% of endothelial cells in the neovasculature formed in response to surgical sponge implantation in mice [18, 19] and may also form vascular mural cells [20]. Increased EPC numbers in the periphrery at the time of MI are associated with improved outcome [21]. On the other hand, monocytosis at the time of MI is associated with an increased left ventricular end-diastolic volume and a reduced ejection fraction [22].

Our findings are in agreement with previously published data about fresh thrombectomy specimens demonstrating the prominent presence of von Willebrand factor at the sites of platelet accumulation, presence of tissue factor and platelets at the sites of deposition of fibrin fibrils [23]. The authors of that study also reported the presence of CD16-, CD45- and CD34-positive cells in the thrombectomy specimens and a weak positive correlation between the number of inflammatory cells involved in the unit area of coronary thrombi specimen and the time of collection of the specimens after the onset of chest pain [23]. More recently, using dye-staining angioscopy, Uchida and colleagues showed *in vivo* that acute coronary syndrome patients can have predominantly fibrin-rich thrombi that have a low platelet content [24], underscoring the variability in cellular content of thrombi causing unstable angina and acute MI.

Our experiments with DMEM culture medium suggest that the cells with progenitor markers were already pre-existing in the thrombectomy specimens. Our experiments with cells in culture using serum-poor stem cell media also suggest that some endothelial progenitor cells are contained in the thrombectomy specimens and persist for prolonged periods of time in culture, but we cannot exclude the possibility that these were mature cell lines, which were re-programmed under low serum stem cell medium conditions throughout 12 months.

In summary, out data suggest that intracoronary thrombi harvested at the time of primary percutaneous intervention from patients with acute myocardial infarction contain various amounts of platelets, fibrin, red and white blood cells as well as cholesterol crystals. The cells are mostly of mesenchymal lineage and include cells with features of endothelial and of endothelial progenitor cells. These findings provide insights into the pathogenesis of plaque rupture, of thrombus lysis and remodelling and of vascular repair.

Conflict of interest

The authors confirm that there are no conflicts of interest.

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