Leukocyte CD11a expression and granulocyte activation during experimental myocardial ischemia and long lasting reperfusion

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J Lantos, L Grama, T Orosz, G Temes, E Rőth. Leukocyte CD11a expression and granulocyte activation during experimental myocardial ischemia and long lasting reperfusion. Exp Clin Cardiol 2001;6(2):72-76.

BACKGROUND: Myocardial ischemia and reperfusion are accompanied by leukocyte activation and expression of surface adhesion molecules, which induce pathological interactions between endothelial cells and circulating neutrophils, leading to tissue damage. While the dynamics of these processes have been well defined during acute reperfusion, there is very little information regarding long lasting reperfusion.
OBJECTIVES: To investigate neutrophil granulocyte (PMN) activation and the CD11a expression of leukocytes during myocardial ischemia and reperfusion for four weeks.
ANIMALS AND METHODS: The left anterior descending coronary artery was occluded for 1 h in six dogs, followed by reperfusion for four weeks. Peripheral blood samples were collected before the operation, at the end of ischemia, at 5 and 60 min of reperfusion, and on postoperative days 1, 2, 3, 7, 14, 21 and 28. Sham operation on four dogs served as control. Leukocyte expression of CD11a was measured by flow cytometry. Superoxide radical production of isolated PMNs was determined spectrophotometrically.
RESULTS: Granulocyte CD11a expression increased while the superoxide radical-producing capacity decreased significantly by the third postoperative day. Sham operation produced similar alterations in these parameters during the first postoperative week. From the second postoperative week, however, granulocyte radical production and adhesion molecule expression were higher in the ischemic animals.
CONCLUSIONS: The exhaustion of PMN radical production and maximal CD11a expression during the first postoperative week are probably due to the surgical trauma caused by thoracotomy, but increased granulocyte function during later reperfusion indicates prolonged healing of injured myocardium.

Key Words: *Granulocyte activation; Heart; Ischemia; Leukocyte CD11a expression; Reperfusion*

Myocardial ischemia and subsequent reperfusion are associated with inflammatory reactions. Activation of neutrophil granulocytes and their adhesion to endothelial cells is largely responsible for mediating local inflammation, plugging of microvasculature and the 'no reflow' phenomenon (1). This process is described as reperfusion injury. Acti-

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vated granulocytes produce a large amount of superoxide radicals by the NADPH enzyme system (2) and express adhesion molecules on their surface membrane. The cellular functions and interactions of leukocytes with vascular endothelial cells are dependent on these surface adhesion molecules. While leukocyte rolling and margination to the endothelium are determined mainly by selectins, the subsequent firm adhesion is mediated by the $_2$-integrin complex (3). The leukocyte integrin family comprises three / heterodimer membrane glycoproteins sharing a common -subunit, CD18, with a distinct -subunit (CD11a, Cd11b, CD11c) (4). The heterodimer glycoprotein Cd11a/CD18 is known as the lymphocyte function-associated antigen-1 (LFA-1), which is on all leukocytes, but is of special interest on lymphocytes (5). Intercellular adhesion molecule (ICAM)-1 is a ligand for LFA-1, and other molecules such as ICAM-2 and ICAM-3 can also bind to LFA-1 (6), but only CD11a has been shown to bind to ICAM-2 and ICAM-3 (7,8). Previous studies have found increased $_2$-integrin expression in circulating blood neutrophils or a beneficial effect of monoclonal antibodies against it during acute experimental myocardial ischemia and early reperfusion (9-13) in isolated heart model (14) and during coronary artery bypass surgery (15). Whereas anti-CD18 are most effective in blocking neutrophil-dependent inflammation in the lung, contributions of CD11a and CD11b seem to be different (6). Leukocytes are involved not only during early tissue damage, but also during healing of the infarcted myocardium, which may take several weeks or more. In the present study the time course of CD11a expression and the dynamism of stimulated superoxide radical-producing capacity of neutrophil granulocytes were investigated after myocardial ischemia for 1 h followed by reperfusion lasting one month.

ANIMALS AND METHODS

Adult mongrel dogs of either sex (n=10) with an average body weight of 17.3±1.7 kg were fasted overnight, and premedicated with droperidol (1.5 mg/kg), fentanyl (0.03 mg/kg) and atropine (1 mg). Anesthesia was induced with 20 to 50 mg/kg thiopental sodium (Trapanal, BYK Gulden, Germany) and maintained with 0.5% to 1% halothane in 70% nitrous oxide and 30% oxygen gas mixture. Pipecuronium 0.05 mg/kg (Arduan, Richter Gedeon Ltd, Hungary) was given to the animals as a muscle relaxant before thoracotomy. The animals were ventilated with positive pressure (Eupulm-4 KA-2 Medicor, Hungary), maintaining an expiratory pressure of 5 to 10 cm H_2O with a trap to prevent atelectasis.

The right femoral artery was cannulated for aortic pressure recording (Sirecust 1260, Siemens, Germany) and blood sampling for blood gas analysis. Electrocardiogram was also recorded on the same polygraph. Arterial blood pH, and partial pressures of oxygen and carbon dioxide were monitored at selected intervals by an automatic blood pH/gas analyzer (OP-216, Radelkis Ltd, Hungary). They were maintained within the normal physiological range by adjusting the respiratory rate or oxygen flow, or by intravenous infusion of 155 mM $NaHCO_3$ when necessary. The right femoral vein was also cannulated for peripheral blood sampling.

Animals were divided in two groups: group 1 (n=6) with 1 h ligature of the left anterior descending coronary artery (LAD) and four-week reperfusion; and group 2 (n=4), which were sham-operated animals. In group 1, left thoracotomy was performed at the fourth intercostal space under sterile conditions, the lung was retracted, and the pericardium was incised. A 2 to 5 mm segment of the LAD was dissected from the surrounding tissue just distal to the first diagonal branch, and a suture thread was passed under it as a means of ligature. Following heparin administration (200 IU/kg) the LAD was ligated for 60 min followed by reperfusion for four weeks. Hemodynamic parameters were recorded continuously during the day of operation, and blood samples were taken before thoracotomy, at the end of a 60 min LAD ligature, and at the acute (5 and 60 min), subacute (one to three days) and chronic periods (one, two, three and four weeks) of reperfusion. The chest of each animal was closed during the early reperfusion period. Following the 60 min blood sampling, 20 mg piroxicam (Hotemin, EGIS, Hungary) was given intramuscularly for postoperative pain relief, and 1 g cefotaxime (Claforan, Aventis Pharma, France) was given intravenously.

In group 2, the same procedure except for the LAD occlusion was carried out on four sham-operated dogs. On the day of surgery blood samples were taken before and after 1 and 2 h of thoracotomy, followed by the same protocol as in group 1.

Cell counting: A Minitron (Diatron Ltd, Hungary) hematological analyzer was used for blood cell counts.

Measurement of the free radical-generating capacity of polymorphonuclear granulocytes: Blood containing EDTA was sedimented with 6% dextrane at 37°C for 1 h. After the supernatant was drawn off, hypotonic hemolysis was performed with 0.8% NH_4Cl to remove erythrocytes (16). The white pellet obtained after repeated centrifugation contained various types of leukocytes. This fraction was used for immunofluorescence staining and flow cytometric analysis. The polymorphonuclear granulocytes (PMNs) were separated thereafter by Ficoll-Paque gradient centrifugation (Amersham Pharmacia Biotech AB, Sweden). Vitality of the cells was determined with vital staining and the cells were counted. The spontaneous and phorbol-miristate-acetate-stimulated superoxide radical production of the PMNs suspended in glucose-containing Dulbecco buffer was measured spectrophotometrically at 535 nm in the presence of 0.1 mM ferricytochrome c. Protein was determined according to Bradford's method.

Measurement of leukocyte CD11a expression: Cell surface CD11a receptors were detected by direct immunofluorescence evaluated by flow cytometry. EDTA-anticoagulated venous blood was sedimented by dextrane as described above. Two hundred microlitres of the whole leukocyte fraction diluted to 3×10^6 cells/mL was used for immunofluorescence staining by 10 µL of fluorescein isothyocyanate (FITC)-conjugated CD11a mouse antihuman monoclonal an-

TABLE 1
Peripheral white blood cell count (WBC) and free radical-producing capacity of polymorphonuclear granulocytes (PMNs) in dogs subjected to experimental ischemia and reperfusion and in sham-operated controls

	WBC ($\times 10^9$ cells/L)		PMNs (nmol $O_2^{-\cdot}$/min/10^6 PMN)	
	LAD ligature (60 min)	Sham	LAD ligature (60 min)	Sham
Before thoracotomy	6.9±0.7	6.6±1.3	15.6±2.9	13.4±2.7
LAD ligature (60 min)	7.0±1.2	6.0±1.2	13.9±3.1	10.8±2.0
Reperfusion				
5 min	7.5±1.0		11.6±2.3	
60 min	8.0±0.6	6.5±1.0	12.4±1.5	10.2±1.8
1 day	32.3±5.2**	27.9±1.6***	17.1±3.1	14.1±1.0
2 days	30.1±2.0***	19.8±1.3***	14.2±4.2	11.5±0.4
3 days	22.6±3.4*	18.9±1.3**	7.7±1.8*	7.2±1.1
7 days	19.4±2.7*	16.6±0.7**	14.8±2.2	9.5±3.1
14 days	14.8±0.8***	17.2±1.9*	13.7±1.8	9.6±2.7
21 days	14.7±2.1*	12.3±1.1	17.1±2.6	9.9±2.7
28 days	17.3±2.6*	10.8±0.7*	18.4±3.9	10.1±1.8

**P<0.05, **P<0.02, ***P<0.01 versus before thoracotomy. LAD Left anterior descending coronary artery*

TABLE 2
Mean channel CD11a fluorescence intensity (arbitrary units)

	Granulocytes		Lymphocytes	
	LAD ligature (60 min)	Sham	LAD ligature (60 min)	Sham
Before thoracotomy	68.4±8.5	65.3±5.9	89.4±9.5	81.0±6.5
LAD ligature (60 min)	54.2±6.1	64.7±6.6	63.1±8.5***	75.0±6.1
Reperfusion				
5 min	59.7±4.1		65.0±4.1*	
60 min	56.5±2.4	61.4±7.9	67.4±8.1	77.0±12.3
1 day	61.9±3.6	60.5±9.8	90.2±13.4	85.6±19.0
2 days	79.1±14.2	81.6±5.5	85.5±15.1	83.3±12.5
3 days	94.2±6.3*	94.8±11.4	85.7±7.7	86.1±11.2
7 days	62.3±4.5	66.3±4.7	84.5±1.5	85.6±9.6
14 days	76.5±2.3	65.8±4.6	90.4±4.6	81.7±12.3
21 days	74.4±4.4	65.2±5.0	87.9±5.3	89.2±13.5
28 days	77.7±3.5	61.7±6.4	92.7±7.5	78.3±8.0

**P<0.05, ***P<0.01 versus before thoracotomy. LAD Left anterior descending coronary artery*

tibody with cross-reactivity for dog leukocytes (Pharmingen 30444X, USA) for 25 min on ice and washed twice before analysis. Mouse immunoglobulin G_1, isotype control was used to determine the nonspecific background fluorescence. Cell immunofluorescence and light scatter data were acquired on a FACSCalibur (Becton Dickinson, USA) flow cytometer. Ten thousand events were acquired for each sample. Binding of FITC-labelled CD11a antibody to neutrophils and lymphocytes was quantified as both the percentage of cells exhibiting specific FITC fluorescence and the mean channel fluorescence in arbitrary units that exceeded nonspecific background fluorescence.

Statistical evaluation: Data are expressed as mean ± SEM. The significance of the differences between the control value and the values measured during ischemia and reperfusion was calculated by paired Student's *t* test. Significance of the differences between the similar data on the ischemic and sham-operated animals was calculated by unpaired Student's *t* test for two populations. The differences were considered significant for $P< 0.05$, $P<0.02$, $P<0.01$ and $P<0.001$.

RESULTS

Peripheral leukocyte counts: Mean peripheral leukocyte counts are shown in Table 1. The baseline values hardly changed during ischemia and the acute reperfusion phase. However, there was a progressive increase in peripheral leukocyte count in both groups by the first postoperative day. The maximal value was seen on the first day followed by a gradual decrease, but in the case of ischemic animals it had increased again by the end of the observation period. Flow cytometric analysis data showed that the number of circulating lymphocytes decreased during ischemia and the acute reper-

fusion phase, and remained lower than that in controls during the first week of reperfusion in group 1. In group 2, however, the number of lymphocytes decreased only on the day of operation. The massive increase in peripheral leukocyte count resulted from the significant increase in granulocytes in both groups.

Peripheral blood neutrophil activation: The mean values of stimulated superoxide radical production of the PMNs are shown in Table 1. In group 1, there was a slight decrease in PMN radical production during ischemia and the acute reperfusion phase compared with controls. After a transient increase during the first day of reperfusion, a marked depletion appeared, which was significant by the third day of reperfusion ($P<0.05$ versus control). The radical-producing capacity of PMNs was slightly increased during the third and fourth week of reperfusion. The tendency for change in PMN radical-producing capacity was similar in sham-operated animals during the first three days following surgery, but from the first week it was in the normal range.

CD11a expression of leukocytes: Table 2 shows the values of the mean channel of fluorescence intensity for CD11a antibody on granulocytes and lymphocytes. At baseline CD11a expression was higher on lymphocytes than on granulocytes. CD11a expression decreased significantly on lymphocytes during ischemia ($P<0.01$ versus control) and the acute reperfusion phase ($P<0.05$) in ischemic animals, while it hardly decreased in the sham-operated group. There was only a moderate decrease in granulocyte CD11a expression at this time. The lymphocyte CD11a expression normalized from the first postoperative day in both groups. Granulocyte CD11a expression, however, started to rise from the second day, reaching its maximal value on the third day of reperfusion in both groups ($P<0.05$ versus control in ischemic animals). However, after a transient decrease in the first postoperative week, CD11a expression of the granulocytes was higher than the preischemic values during late reperfusion in group 1. In the sham-operated animals CD11a expression of granulocytes normalized from the first postoperative week.

DISCUSSION

The main cause of tissue damage during myocardial ischemia is the metabolic derangement of ATP and the consequent energy depletion of cardiac cells (17). However, the restitution of blood flow to the previously ischemic areas may itself contribute to additional myocardial tissue injury by the increased radical production (18-21) and the activation of neutrophil granulocytes (22), followed by a marked influx of leukocytes to the injured area (23). This inflammatory reaction might extend the previous ischemic damage and complicate cardiac injury leading to damage of potentially viable myocardium. While leukocytes play an important part in the healing of myocardial infarct that may require a longer period, it seems worthwhile to investigate the changes in the function of leukocytes during chronic reperfusion following myocardial ischemia.

The acute activation of granulocytes can be characterized by radical production by their NADPH enzyme system (2,16). Increased superoxide radical production during early reperfusion inactivates nitric oxide-producing peroxinitrite, inducing also the respiratory burst (24). Inflammatory mediators such as leukotriene B4 and platelet-activating factor also induce the respiratory burst (25,26). There is increased adhesion molecule expression on activated granulocytes, which have a basic role in the leukocyte endothelial cell interaction, namely rolling, firm adhesion and migration of granulocytes to the extravascular tissue (4). The plugging of microvasculature and subsequent inflammation are the consequences of these processes (1,22,27).

In this study, changes in leukocyte count, granulocyte activation state and expression of CD11a adhesion molecule on lymphocytes and granulocytes in peripheral blood samples were investigated. Coronary ligature for 60 min was induced in an open chest model under sterile conditions. The chest was closed during early reperfusion, and the state of the animal was followed for four weeks. While prolonged surgical intervention is invariably associated with a marked increase in neutrophil count and overall leukocyte activation, the effect of sham operation was also investigated.

In this preliminary study we could not differentiate granulocyte and monocyte populations by flow cytometric measurement; thus, the granulocyte data may include monocytes. We observed high level leukocytosis from the first postoperative day in both experimental groups. Flow cytometric analysis proved that the marked elevation of leukocyte counts was due to an increased number of granulocytes (28).

Superoxide radical production by granulocytes showed a tendency to decrease during ischemia and acute reperfusion. On the first day of reperfusion it rose transiently, probably due to the liberation of inflammatory mediators. Granulocyte activation dropped significantly by the third day of reperfusion, suggesting that, despite the significant rise in the number of circulating cells, most of them might have been exhausted or premature.

Flow cytometric analysis showed a decreasing tendency in granulocyte CD11a expression during ischemia and early reperfusion, but it started to rise from the second postoperative day. The peak of CD11a expression was seen on the third day of reperfusion, at the same time as the lowest activity of neutrophils was measured to produce superoxide radical. Similar data were obtained in sham-operated animals, showing that the surgical stress caused by thoracotomy overwhelms the acute effect of myocardial ischemia and reperfusion. From the second postoperative week, however, higher adhesion molecule expression was observed in animals that were subjected to myocardial ischemia. This finding may reflect that the healing of myocardial infarction may require a longer period of time and that there are cellular changes even after three to four weeks of reperfusion. This phenomenon was also found in our earlier experiments, in which oxidative stress was investigated in a similar model (21).

On the basis of these data we can hypothesize that in the acute postoperative period the first step is activation of the

membrane-based NADPH-oxidase of circulating neutrophils. The second step is the expression of adhesion molecules on the surface of these cells, which are responsible for the altered behaviour of neutrophils (rolling, adherence and migration). This is in accordance with the result of the granulocyte activation state, namely that increasing the adhesive ability of granulocytes is expected to lead to adherence of the active cell to the injured area. From this point of view the third postoperative day seems to be critical following major surgical intervention.

CD11a expression on the surface of lymphocytes decreased markedly during ischemia and acute reperfusion, and normalized by the first postoperative day. This suggests that open chest surgical trauma, as well as myocardial ischemia and reperfusion, also influenced the activation state of lymphocytes, but it was only a transient event (5,29).

CONCLUSIONS

There was evidence that during myocardial ischemia and reperfusion in an open chest model the expression of adhesion molecules depends on the type of cells. The decrease in CD11a expression on lymphocytes is the first step of this process followed by increased granulocyte CD11a expression lasting up to the third postoperative day. A parallel decrease in granulocyte radical-producing capacity may confirm the presence of granulocyte-endothelial cell interaction in the injured area. Data obtained in sham-operated animals showed that the surgical stress caused by thoracotomy overwhelms the acute effect of myocardial ischemia and reperfusion in the first postoperative week. Later on, however, a difference was present between the two groups in the expression of granulocyte CD11a expression and radical-producing capacity, which is mirrored in increased granulocyte activation. Monitoring these parameters may be useful in the follow-up of patient recovery following myocardial infarction.

ACKNOWLEDGEMENTS: This work was supported by the Hungarian Scientific Research Fund OTKA No T 25846; ETT 369-02.

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