

Apoptosis of Lymphocytes and Sialic Acid Plasma Level in Patients Undergoing Cardiac Surgery

Anna WYSOCKA, Anna KORYCIŃSKA, Michał DRAGAN, Henryk BERBEĆ, Jacek ROLIŃSKI and Janusz STAŻKA

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Increasing evidence shows that extensive tissue trauma and surgical stress are related to physical alterations of cells and cell death. It was previously reported that total sialic acid (SA) plasma concentration is elevated in patients undergoing coronary artery surgery. Shedding or secreting of SA from the cell membrane surface or releasing intracellular SA may induce apoptosis. It is possible that the terminal SA residues of carbohydrate moieties facilitate recognition and removal of apoptotic cells by phagocytes. The aim of the present study was to estimate the dynamic changes in rate of apoptosis of lymphocytes and total sialic acid plasma level during coronary artery surgery. In 17 patients undergoing coronary artery bypass grafting surgery plasma total SA concentration was measured and the percentage of apoptotic lymphocytes was determined before operation, after aorta clamping, after the end of operation and at 6, 18, 30 and 48 h after operation. Plasma total SA concentration decreases after aortic clamping and then increases gradually during a 48 hr observation period. The percentage of apoptotic cells increases during and after surgery with the exception of a sample taken at 18 hours after operation. The findings indicate the bimodal character of apoptosis and dynamic increase in total SA plasma level, which may be considered a result of mechanical damage taken place during operation or inflammatory response to surgical trauma.

Key words: Apoptosis, sialic acid, coronary artery bypass graft.

Anna WYSOCKA, Henryk BERBEĆ, Department of Biochemistry and Molecular Biology Skubiszewski's University Medical School, W. Chodźki 1, 20-093 Lublin, Poland.
E-mail: hanah5@poczta.onet.pl

Anna KORYCIŃSKA, Michał DRAGAN, Jacek ROLIŃSKI, Department of Immunology Skubiszewski's University Medical School, Jaczewskiego 8, 20-954 Lublin, Poland.

Janusz STAŻKA, Department of Cardiosurgery Skubiszewski's University Medical School, Jaczewskiego 8, 20-954 Lublin, Poland.

Apoptosis or programmed cell death is a conserved process designed to remove damaged cells from an organism without inducing inflammation. Apoptosis allows cells to die in a strictly controlled and organized manner. There are different phases of this process. During the induction phase, the cells receive a signal that will elicit the apoptotic process. This phase is initiated by a number of external and internal factors such as toxins, DNA damaging agents, glucocorticosteroids or oxygen free radicals. The induction phase is mediated by different active proteins such as NF- κ B, TNF or the Fas-Fas ligand complex (PALLARDY *et al.* 1997). During the effector phase the apoptotic machinery is irreversibly set in motion through the activation of caspases. In a degradation phase, cells committed to die by apoptosis display cell-surface markers that allow for their recognition and removal by phagocytic cells. One of important

markers involved in this phase is phosphatidylserine, which is exposed to the outer leaflet of the plasma membrane. Another important action is the removal of sialic acid from the cell surface. At the end specific endonucleases are activated, leading to DNA fragmentation with characteristic DNA strand breaks and chromatin condensation which are morphological manifestations of the late stage of apoptosis (GREEN *et al.* 1995).

Sialic acid (N- acyl-neuraminic acid) is a component of glycoproteins and glycolipids, derivatives of which are usually located on the outer side of cell membranes. Sialic acid has been recognised as the most essential monosaccharide with respect to biological function when present on cell membranes. This highly negatively charged carbohydrate moiety may influence cell growth and cell to cell interactions (ZELLER *et al.* 1992). An increase in plasma sialic acid concentration is connected

with intensified synthesis and release of sialoglycoproteins by the liver caused by some type of acute phase reaction in the inflammatory process. It may also be a result of shedding of sialic acid from the cell surfaces or the release of intracellular sialic acid (SCHAUER 1995). Several publications (e.g. BERBEC *et al.* 1999; PASZKOWSKA *et al.* 1997, 1998) have confirmed that the majority of patients with malignant tumors and inflammatory diseases have an elevated concentration of SA in blood. Recently, it has been observed that glycosphingolipids containing different amounts of sialic acid moieties are positive regulators of programmed cell death (ZHOU *et al.* 1997).

There are several studies supporting the idea that extensive tissue trauma as well as surgical stress may affect immune functions by the process of apoptosis (DELOGU *et al.* 2000; RAO *et al.* 1997). One of the most important factors triggering pro-

grammed cell death may be mechanical damage of lymphocytes during coronary artery bypass grafting (CABG) with extracorporeal circulation. Another factor that may be associated with the release of SA is systemic inflammation, irreversibly connected with cardiac surgery.

The aim of the present study was to estimate dynamic changes in rate of apoptosis of lymphocytes and total sialic acid plasma level during coronary artery surgery.

Material and Methods

Seventeen patients (13 men, 4 women) with a median age of 61 years (range 43-73), undergoing elective coronary artery bypass grafting surgery with extracorporeal circulation were included in the study. The risk of operation was evaluated ac-

Table 1
Statistical analysis of the correlation between aortic cross-clamping time and percentage of apoptotic cells

	Stage of experiment						
	1	2	3	4	5	6	7
Correlation coefficient (r)	-0.08	-0.06	0.45	0.61	0.58	0.13	0.15
Statistical significance (P)	0.77	0.83	0.1	0.01	0.01	0.65	0.59

1—preoperatively, 2—after aortic clamping, 3—after the end of operation, 4—at 6 h, 5—at 18 h, 6—at 30 h, and 7—at 48 h after operation.

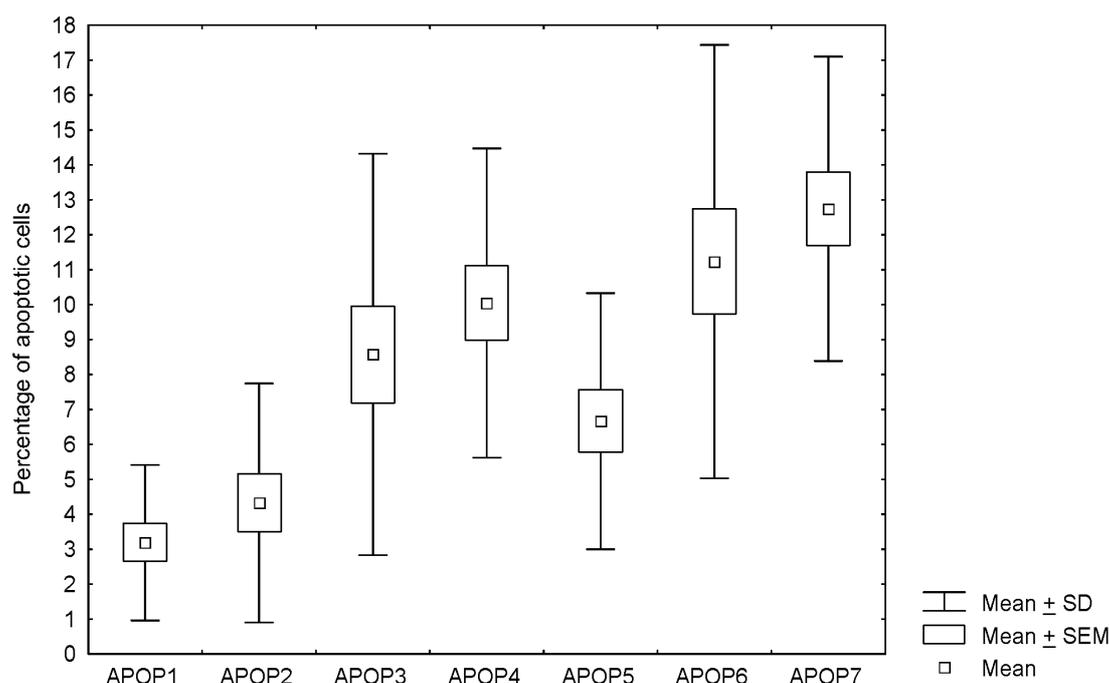


Fig. 1. Percentage (%) of apoptotic cells measured by the FDA method in subsequent stages of the experiment: (1) preoperatively, (2) after aortic clamping, (3) after the end of operation, (4) at 6 h, (5) at 18 h, (6) at 30 h, and (7) at 48 h after operation.

ording to the Euroscore scale (MICHEL *et al.* 2003).

Venous blood samples were obtained before and after coronary bypass grafting at the following moments: (1) preoperatively, (2) after aortic clumping, (3) after the end of operation, (4) at 6 h, (5) at 18 h, (6) at 30 h, and (7) at 48 h after operation. Concentration of total SA was assayed by the modified thiobarbituric acid method of Warren (CROOK *et al.* 1993). The concentration of total plasma protein was assayed by the biuret method. The total sialic acid/protein index expressed in micromol of SA per gram of protein was calculated.

For the evaluation of apoptosis, cells were exposed to fluorescein diacetate (FDA) used in combination with propidium iodide (PI). The samples were analyzed by flow cytometry (FASCalibur, Becton Dickinson, USA) just after intervention.

10 000 cells were collected in each experiment. The gate was set on the population of lymphocytes alive and apoptotic.

Data were statistically analysed using the Software STATISTICA for Windows (Statsoft, Inc. 1993 release 5.1). The Wilcoxon paired test was used to compare mean value differences at each stage of experiment. Correlation was calculated using the Spearman test. P values less than 0.05 were considered significant.

Results and Discussion

The median CABG surgery time was 169 min (range 130-240 min) and the median aortic cross clumping was 46 min (range 28-57 min). The median risk of operation according to Euroscore was

Table 2

Correlation between risk of operation according to the Euroscore and total SA level and between risk of operation and index of SA/ per g of protein

	Stage of experiment							
		1	2	3	4	5	6	7
Total SA	r	0.59	0.57	0.52	0.60	0.51	0.63	0.71
	p	0.01	0.01	0.044	0.009	0.03	0.008	0.003
SA index (sialic acid/g of protein)	r	0.36	0.45	0.56	0.37	0.32	0.37	0.43
	p	0.16	0.06	0.02	0.14	0.20	0.08	0.12

1 – preoperatively, 2 – after aortic clumping, 3 – after the end of operation, 4 – at 6 h, 5 – at 18 h, 6 – at 30 h, and 7 – at 48 h after operation.

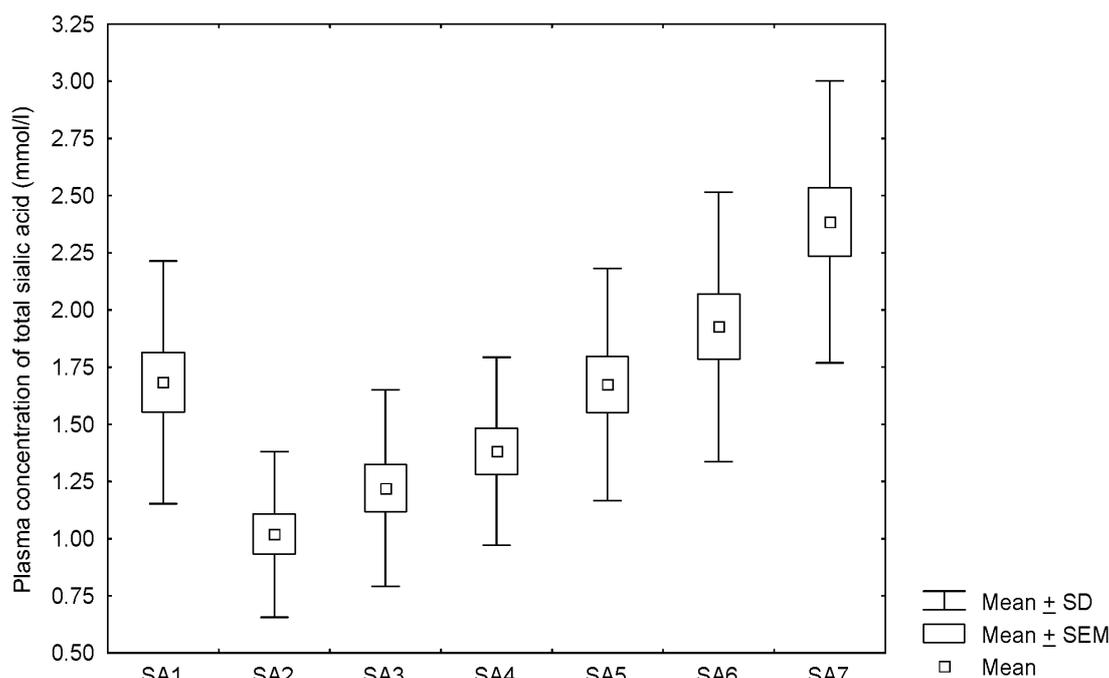


Fig. 2. Plasma level of total sialic acid (mmol/L) in subsequent stages of the experiment: (1) preoperatively, (2) after aortic clumping, (3) after the end of operation, (4) at 6 h, (5) at 18 h, (6) at 30 h, and (7) at 48 h after operation.

3.94 points (range 0-10). There was a significant correlation between aortic cross-clamping time and percentage of apoptotic cells in the fourth and fifth stages of the experiment (Table 1).

A significant increase in the number of apoptotic lymphocytes in all stages of the experiment was detected in comparison with the preoperative period ($P < 0.001$). Changes in the percentage of apoptotic cells were of bimodal character, i.e. increasing significantly in the second, third and fourth stages of the experiment, while decreasing in the fifth and increasing again in the sixth and seventh stages (Fig. 1). In recent years the link between myocardial ischemia resulting from general anesthesia and neuroendocrine system activation during cardiac surgery and apoptosis has been widely discussed (ANSELMINI *et al.* 2004). Cardiovascular by-pass surgery is associated with the possibility of mechanical damage of peripheral blood cells during extracorporeal circulation initiated by contact of the large artificial surfaces of the cardiopulmonary by-pass circuit. Investigators focused mainly on the influence of the systemic inflammatory process and ischaemia reperfusion phenomena on myocytes. There have been several experimental studies on animal models, and human myocardial biopsies investigated apoptosis during CABG. In patients undergoing elective CABG surgery, no signs of terminal stages of the apoptotic process were found, but some nuclei changes and swollen mitochondria that indicate that an early apoptotic phase was ob-

served. Swollen mitochondria with decreased mitochondrial intermembrane potential are the first detectable sign of cellular apoptosis, but some changes connected with the exposure of phosphatidylserine on the plasma membrane (measured by the use of annexin V) are also regarded as hallmarks of early apoptosis. It has been confirmed that the activity of certain esterases (e.g. responsible for hydrolysis of fluorescein diacetate - FDA) corresponds to these changes in plasma membrane (FADOK *et al.* 1992). In this study the FDA method was used for the assessment of early apoptosis because it is quick, less expensive and reliable. The duration of cardioplegia et reperfusion correlated positively with changes characteristic for apoptosis. Also, a positive correlation in cardiac hemodynamic parameters and pulmonary capillary wedge pressure was noticed (SCHMIDT *et al.* 2002). An increased percentage of apoptotic myocytes and decreased expression of bcl-2 was found in endomyocardial biopsies from patients that underwent cardiac surgery with short term post-operative mortality in comparison with a group of patients with longer survival (ZORC *et al.* 2003). Apoptosis of myocytes is an inflammation-dependent process that occurs even after the elimination of damaging agents. There are hypotheses suggesting that necrosis of seriously damaged myocytes stimulates the subsequent apoptosis of surviving cells. The reason for this phenomenon is that necrotic cells enhance the inflammatory process by providing local endothe-

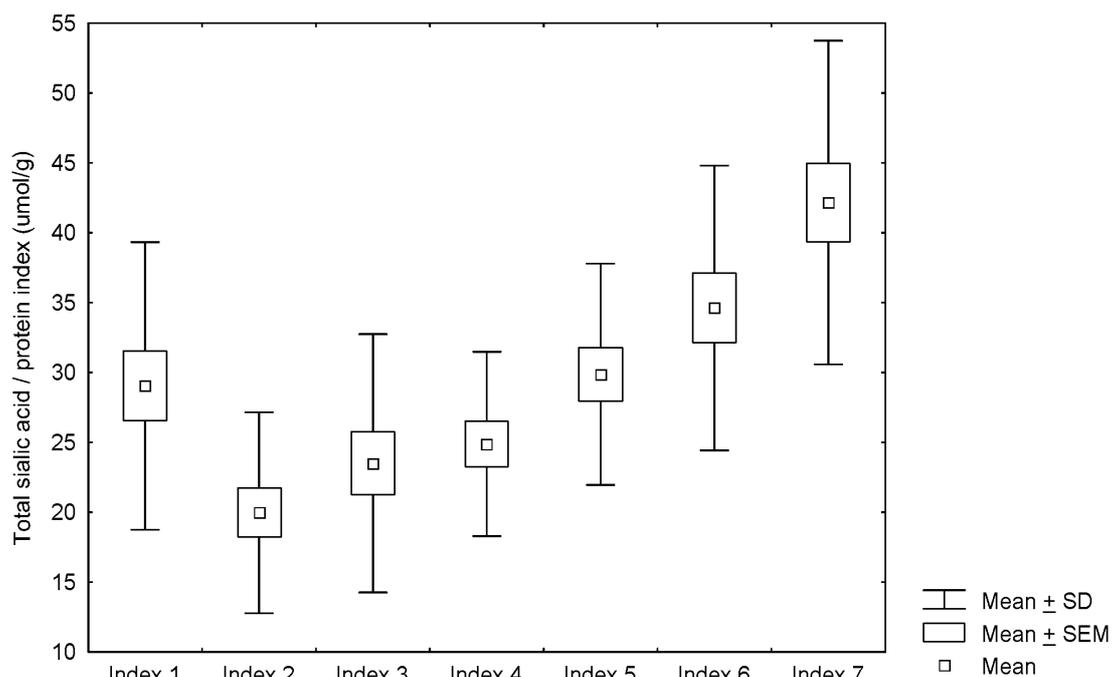


Fig. 3. Total sialic acid/plasma protein index ($\mu\text{mol/g}$) in subsequent stages of the experiment: (1) preoperatively, (2) after aortic clamping, (3) after the end of operation, (4) at 6 h, (5) at 18 h, (6) at 30 h, and (7) at 48 h after operation.

lial activation, monocyte chemoattraction and infiltration. Cytokines such as IL-6 and TNF- α triggering apoptosis are released, providing an increase in serum level of cytochrom c, as well as the level of soluble Fas receptor (PAPARELLA *et al.* 2002). Apart from apoptosis of myocardiocytes, T lymphocytes activated by the inflammatory process also demonstrated apoptotic changes. This reaction is called activation-induced cell death. On the other hand, it was reported that serum obtained from patients undergoing CABG induced apoptosis on cultured endothelial cells, while serum from healthy control subjects or from patients who had other surgical procedures did not show pro-apoptotic activity (BIRDI *et al.* 1997). The present findings confirm the influence of extensive surgical stress and systemic inflammation on the apoptosis process. The pathomechanism of the bimodal character of the rate of apoptosis remains to be clarified. The reason may be the simultaneous activation of different pathways of apoptosis: caspase-dependent and the lately described caspase independent reaction by different pro-apoptotic factors. Like classical apoptosis pathways, caspase independent death programs can be triggered by death receptors in the plasma membrane or by DNA damage, both of which are controlled at the mitochondrial outer membrane and executed by proteolytic enzymes (JAATTELA *et al.* 2003).

The total plasma sialic acid concentration before, during and after CABG surgery are shown in Figure 2. A significant decrease of total sialic acid level after aorta cross clamping was observed ($P < 0.001$) if compared with the preoperative values. In the subsequent stages of the experiment, the level of total sialic acid increases, but remains significantly lower ($P = 0.001$) if the preoperative period is compared to the fourth stage. Total sialic acid plasma concentration in the fifth and sixth stages does not differ significantly from the preoperative period. Patients tested 48 h after cardiopulmonary bypass surgery have significantly higher ($P = 0.001$) plasma sialic acid concentration in comparison with all previous stages of experiment. The sialic acid/protein index decreases significantly ($P < 0.01$) after aorta clamping and remains lower ($P < 0.05$) in the second and third stages of the experiment in comparison with the preoperative period. It increases significantly in subsequent stages to achieve a level similar to the preoperative value at 18 h after operation. The SA/protein index reaches a maximum at 48 h after operation. The correlation coefficient between the risk of operation according to the Euroscore and total SA level and between the risk of operation and the index of SA/ per g of protein is shown in Table 2. Although SA was found to increase in inflammatory processes, including ischaemic cardiovascular disease and myocardial infarction

(WATTS *et al.* 1995; ALLAIN *et al.* 1996), there has been no study investigating the dynamic changes of plasma total SA concentration during cardiac surgery. A few studies have demonstrated that the serum SA level increases in patients with acute myocardial infarction (HAQ *et al.* 1993; GOKMEN *et al.* 2000). During CABG surgery aorta cross-clamping and cardioplegic cardiac arrest induces global ischaemia, so cardiac surgery can be considered a human model of controlled ischaemia similar to that occurring in myocardial infarction. Recently, SA concentration in a coronary sinus after aorta declamping was found as a unique and novel marker useful in the assessment of myocardial cell damage (BERKEN *et al.* 2002). SA was released into the coronary sinus almost simultaneously with commonly used markers of myocardial damage such as creatine kinase, myoglobin and cardiac troponin T within the first minute after aorta declamping. The possible source of increasing plasma SA concentration is the release of intracellular SA as a result of myocardial ischaemia and necrosis. Another is the well established process of the shedding or secretion of SA from the cell membrane surface. Sialidase, an enzyme that is responsible for the removal of SA moieties from the glycoproteins and glycolipids, may contribute to its secretion. Sialidase activity is increased in patients with acute myocardial infarction and with coronary heart disease (HANSON *et al.* 1987; SONMEZ *et al.* 1998). Apart from these processes another source of increasing SA concentration is likely to be associated with increased activity of sialyltransferase (FRASER *et al.* 1980). This enzyme has been reported to be stimulated by platelet-aggregating factors. Exposure of the blood to the extracorporeal circuit activates the contact system and initiates the coagulation cascade. Also, the activation of the complement system, mainly through its alternative pathway by heparin-protamin complexes during ECC, was observed (GU *et al.* 1999). In general, heparin, hypothermia and trauma caused by the interaction with the cardiopulmonary bypass circuit are considered the main triggers for platelet activation.

No correlation between the number of apoptotic lymphocytes and plasma total SA concentration was found.

In conclusion: coronary artery bypass graft operation (CABG) is associated with an increasing percentage of apoptotic lymphocytes. The causes of bimodal phase of apoptosis observed in this study require further experiments. A dynamic increase in total sialic acid plasma level and total sialic acid/protein index is shown in all postoperative stages. A decrease in total sialic acid concentration after aortic cross clamping may result from hemodilution. Both processes, apoptosis and shed-

ding or secretion of sialic acid from cell membranes, may indicate mechanical cell damage that has occurred during extracorporeal circulation or inflammatory response to surgical trauma.

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