

Changes in transforming growth factor β and its receptors' mRNA expression in monocytes from patients with acute coronary syndromes

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Abstract

Introduction: Transforming growth factor β (TGF β) is thought to be a vasoprotective cytokine. Numerous reports confirm its significance in blood and plaques. There is, however, a lack of information on the molecular mechanisms involving TGF β in circulating inflammatory cells in atherogenesis. The aim of the study was to assess gene expression of TGF β and its receptors in monocytes from patients with acute coronary syndromes (ACS) and the effect of standard treatment on the studied genes.

Material and methods: The study was carried out in 32 patients with ACS and 15 healthy subjects. Gene expression of TGF β and receptors TGF β RI and RII was evaluated on day 1 and 5 in the study group and once in controls. The number of mRNA copies isolated from monocytes was assessed by QRT-PCR.

Results: Monocytes of ACS patients showed slightly elevated transcriptional activity of TGF β 1 and its receptors RI and RII genes (0.29 ± 0.043 vs. 0.08 ± 0.020 , $p = 0.05$; 0.071 ± 0.022 vs. 0.036 ± 0.023 , $p < 0.05$; 0.134 ± 0.020 vs. 0.048 ± 0.016 , $p < 0.05$, respectively). After 5-day standard treatment modest reduction of TGF β RI expression was observed. The studied genes' expression was unrelated to EF, myocardial necrosis markers, GRACE score, time from the onset of pain to PCI and angiographic findings. Among risk factors family history of CAD was associated with increased TGF β RI expression. Moreover, the presence of 4 or more classic risk factors correlated with higher TGF β RI expression.

Conclusion: Monocytes of ACS patients demonstrate overexpression of TGF β 1 and its receptors' genes. 5-day standard treatment downregulated the TGF β RI gene but did not affect TGF β 1 and TGF β RII.

Key words: monocytes, gene expression, transforming growth factor β , acute coronary syndromes.

Introduction

Acute coronary syndromes (ACS), including myocardial infarction (AMI) and unstable angina (UA), are among the leading causes of death in Western societies and have become the major concern of clinical cardiology. A number of studies over the past two decades have revealed that ACS is primarily caused by atherosclerotic plaque rupture followed by intravascular thrombosis resulting in total or partial coronary artery occlusion. Many factors responsible for the plaque vulnerability, including

inflammatory factors, coagulation disturbances and endothelial dysfunction, have been described [1]. Recent molecular studies have revealed altered transcriptional activity of many pro- and anti-inflammatory genes involved in plaque formation and rupture, both in atherosclerotic plaque itself and in circulating inflammatory cells in patients with ACS.

Transforming growth factor β (TGF β) is a cytokine engaged in a wide range of physiological and pathological processes [2]. Apart from embryonic development and cell growth control, it participates in wound formation, carcinogenesis, inflammation, and fibrosis. TGF β signalling is mediated by two major receptors: RI and RII – serine/threonine kinase type proteins, which eventually activate TGF β dependent transcription factors [3].

In the pathophysiology of the cardiovascular system TGF β seems to act mostly protectively due to its anti-inflammatory and anti-atherogenic action [4]. It inhibits activation of macrophages and T-lymphocytes, and reduces vascular smooth muscle cell proliferation [5, 6]. In experimental models TGF β inhibits inflammatory cell migration into the subintimal space and stimulates extracellular matrix formation [7, 8]. Although there are some data on the action of TGF β at the protein level, not much is known about the molecular disturbances of TGF β gene transcription in cardiovascular diseases.

Monocytes are one of two major populations of blood derived immune cells and are involved in atherosclerotic plaque formation, growth and rupture by producing numerous inflammation modulating factors. While it is difficult to obtain the plaque infiltrating immune cells, circulating blood cells are a good source of information on the molecular mechanism of the inflammatory reaction in atherosclerosis. Often used for these purposes, peripheral blood mononuclear cells (PBMC), despite some advantages, are a heterogeneous population.

Therefore, the aim of the study was to assess (1) gene expression of TGF β and its receptors in monocytes from patients with ACS, (2) the correlation between expression of the studied genes and classical risk factors of CAD, and (3) the effect of standard treatment on expression of the studied genes.

Material and methods

Patients

The study was carried out on:

- 32 patients with acute coronary syndrome [aged 56.3 \pm 8.2 years (mean \pm SD)] including 14 patients with ST elevation myocardial infarction (STEMI), 9 patients with non-ST elevation myocardial infarction (NSTEMI) and 9 patients with unstable angina, with no history of CAD, and
- 15 sex- and age-matched healthy subjects (C = control group).

In each patient the full blood cell count, leukocyte differentiation, CK (creatinine kinase), CK-MB, troponin I, hsCRP, creatinine, glycaemia and lipid parameters were measured. Patients with previously diagnosed CAD, including those with a history of MI and coronary revascularization, were excluded. Also, chronic inflammatory diseases, chronic heart failure, chronic kidney disease (eGFR < 90 ml/min/1.73 m² calculated with MDRD), as well as diabetes mellitus, were exclusion criteria. All ACS patients underwent primary coronary intervention and were treated according to European Society of Cardiology Guidelines for management in ACS. All subjects received ASA, clopidogrel and statin. ACE inhibitors (82%) and beta-blockers (82%) were administered unless contraindicated (Table III). Control subjects had a standard exercise treadmill test performed (Bruce protocol). Detailed characteristics of studied groups including ACS risk profile are shown in Table I. Written informed

Table I. Baseline characteristics

| Parameter | Study Group | | | | Control Group (n = 15) |
|------------------------------|-----------------------|-----------------|----------------|----------------|---------------------------|
| | ACS (all) (n = 32) | Subgroups | | | |
| | | STEMI (n = 14) | NSTEMI (n = 9) | UA (n = 9) | |
| Age (years \pm SD) | 56.3 \pm 8.2 | 56.6 \pm 10.2 | 56.6 \pm 4.4 | 55.3 \pm 8.6 | 51 \pm 8.6 |
| Sex, M/F | 26/6 | 12/2 | 7/2 | 7/2 | 9/6 |
| Family history, n (%) | 10 (31%) | 3 (21%) | 3 (33%) | 4 (44%) | 5 (33%) |
| Smoking, n (%) | 24 (75%) | 8 (57%) | 7 (77%) | 7 (77%) | 2 (13%) |
| Hypertension, n (%) | 21 (66%) | 10 (71%) | 5 (55%) | 6 (66%) | 0 |
| Overweight, n (%) | 20 (63%) | 7 (50%) | 6 (66%) | 7 (77%) | 5 (33%) |
| BMI [kg/m ²] | 26.6 \pm 0.56 | 26.2 \pm 1.05 | 26.6 \pm 1.0 | 27.3 \pm 0.8 | 24.5 \pm 0.55 |
| Hypercholesterolaemia, n (%) | 17 (53%) | 8 (57%) | 4 (44%) | 5 (55%) | 3 (20%) |
| Hypertriglyceridaemia, n (%) | 5 (16%) | 1 (7%) | 1 (11%) | 3 (33%) | 1 (7%) |

Table II. Clinical characteristics of ACS

| | Study Group | | | |
|--|--------------------|----------------|----------------|-------------|
| | ACS (all) (n = 32) | STEMI (n = 14) | NSTEMI (n = 9) | UA (n = 9) |
| Time from the onset of chest pain [h] | 7.2 [1-24] | 4.2 [1-10] | 8.9 [2-24] | 10.1 [2-24] |
| One-vessel / multivessel disease [n/n] | 21/11 | 10/4 | 6/3 | 5/4 |
| PCI Target vessel [LAD/LCX/RCA] | 14/7/11 | 6/2/6 | 5/4/0 | 3/1/5 |
| Ejection Fraction [%] | 53 ±1.4 | 50 ±2.0 | 55 ±2.0 | 56 ±3.0 |
| Myocardial necrosis markers: | | | | |
| CK [UI/l] | 1471 ±365.6 | 27834 ±674.8 | 773 ±233.5 | 124 ±8.8 |
| CK-MB [UI/l] | 149 ±31.4 | 256 ±55.8 | 111 ±32.4 | 19.3 ±1.33 |
| Troponin I [ng/ml] | 10.5 ±2.18 | 19.6 ±3.17 | 7.9 ±3.19 | < 0.01 |
| GRACE: In hospital death [% range] | – | 16-25 | 12-18 | 6-12 |
| GRACE: 6-month death or MI [% range] | – | 2-6 | 1-4 | 1-4 |
| eGFR (MDRD) | 101.5 ±13.4 | 103.9 ±15.4 | 98.7 ±10.4 | 100.0 ±13.0 |

Table III. Pharmacological treatment of ACS patients

| | Number of pts treated [%] | | | Average dose/day [mg] | | |
|----------------|---------------------------|----------|----------|-----------------------|--------|-----|
| | STEMI | NSTEMI | UA | STEMI | NSTEMI | UA |
| ASA | 14 (100%) | 9 (100%) | 9 (100%) | 128 | 108 | 116 |
| Clopidogrel | 14 (100%) | 9 (100%) | 9 (100%) | 75 | 75 | 75 |
| Statins: | 14 (100%) | 9 (100%) | 9 (100%) | – | – | – |
| Simvastatin | 3 | 1 | 3 | 26 | 20 | 20 |
| Atorvastatin | 11 | 8 | 6 | 38 | 37.5 | 33 |
| Beta-blockers | 13 (93%) | 8 (89%) | 7 (78%) | – | – | – |
| Metoprolol | 10 | 5 | 6 | 57 | 45 | 46 |
| Bisoprolol | 3 | 3 | 1 | 4.6 | 2.9 | 2.5 |
| ACE inhibitors | 11 (79%) | 8 (89%) | 9 (100%) | – | – | – |
| Ramipril | 8 | 8 | 8 | 3.75 | 4.1 | 3.4 |
| Quinapril | 3 | 0 | 1 | 15.8 | – | 5 |
| Other: | | | | | | |
| Spironolactone | 7 (50%) | 2 (22%) | 0 | 32 | 37.5 | – |

consent was obtained from each participant of the study.

Blood samples were collected from the basilic vein into tubes containing EDTA. The samples were obtained from patients within 12 hours after the onset of chest pain and again after 5 days of standard treatment, including PCI. Time from the onset of chest pain to first blood sampling is indicated in Table II. In all cases the blood (day 1 sample) was drawn before PCI and before introducing full pharmacological treatment. Before the collection patients could only receive emergency ACS treatment e.g. ASA, heparin, nitrates and clopidogrel). In the control group blood was taken once. Upon blood sampling, the monocytes were

immediately isolated using RosetteSep (StemCell Technologies, USA) monocyte separation kits. The procedure was performed according to the producer's manual. Purity of isolated monocytes was > 96%. After isolation monocytes were stored at –70°C in RNAlater (Qiagen, USA) stabilization reagent until RNA extraction.

RNA extraction

Total RNA was extracted from monocytes using a modified Chomczynski and Sacchi method. All extracts were treated with DNase I to avoid contamination by genomic DNA. The RNA extracts were qualitatively evaluated by electrophoresis

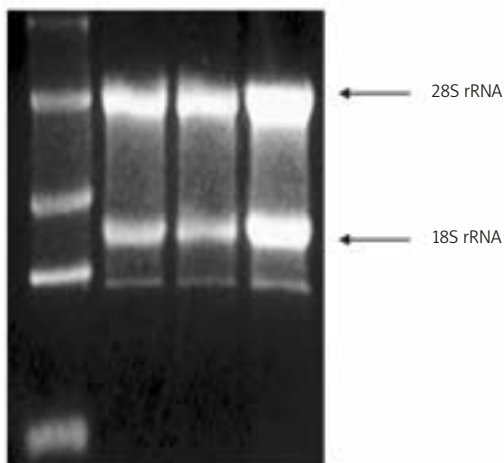


Figure 1.

(sample electrophoresis shown in Fig. 1) in 1% agarose gel stained with ethidium bromide, and quantified spectrophotometrically (Gene Quant II by Pharmacia).

Primers

Primers and probes for amplification of TGFβ1 and its type I (ALK5), and II receptors were determined using the Primer Express Version 1.0 computer program (PE Applied Biosystems, USA), and their sequence homologies were checked using the GenBank database (<http://www.ncbi.nlm.nih.gov/ix/genbank>).

Primer sequences were (f – forward; r – reverse):
 TGFβ (f) 5' TGGAACCGGCCTTCTGCTTCTCATG 3'
 (r) 5' GCGGAAGTCAATGTACAGCTGCCGC 3'
 TGFβ receptors
 TGFβ RI
 (f) 5' ACTGGCAGCTGTATTGCTGGACCAG 3'
 (r) 5' CCTGAGCCAGAACCTGACGTTGTCATATCA 3'
 TGFβ RII (f) 5' GGCTCAACCACCAGGGCATCCAGAT 3'
 (r) 5' CTCCCGAGAGCCTGTCCAGATGCT 3'

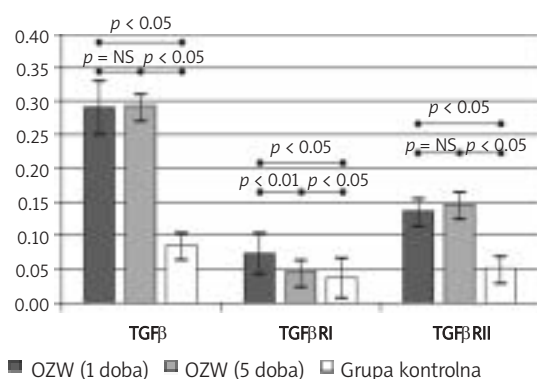


Figure 2. Gene expression of TGFβ-1 and its receptors RI i RII in peripheral blood monocytes

The β-actin gene was used as the endogenous control and was amplified simultaneously with assessed genes. Primer sequences used for β-actin amplification were:

(f) 5' TCACCCACATGTGCCCATCTACGA 3'
 (r) 5' CAGCGGAACCGCTCATTGCCAATGG 3'

Real-time PCR (QRT-PCR)

In order to determine the number of mRNA copies for TGFβ and its type I and II receptors, quantitative reverse transcriptase polymerase chain reaction (QRT-PCR) was performed using QuantiTect SYBR Green RT-PCR. Cycling conditions were as follows: RT: 60°C 30 min; polymerase activation: 94°C for 15 min; PCR: 94°C for 15 s and 53-68°C for 30 s, 40 cycles; final elongation: 72°C for 10 min. QRT-PCR specificity was confirmed experimentally by PAA electrophoresis, amplimers' melting temperature measurement using ABI PRISM7000 (SYBR Green RT-PCR Kit), and sequence analysis using ABI PRISM 377 DNA Sequencer (PE Applied Biosystems). The results are shown as studied gene to β-actin mRNA level ratio.

Statistical analysis

All values were expressed as means ± standard error (AVG ± SEM). Differences were considered to be significant at $p < 0.05$. In order to check the normality of the distribution, the Shapiro-Wilk test was performed. In case of a normal distribution Student's t-test was performed; otherwise the Mann-Whitney U test was used. To compare two related variables the Wilcoxon test was used. Analysis of correlation was performed using the Spearman test.

The study was accepted by the Ethics Committee of the Medical University of Silesia.

The investigation conforms to the principles outlined in the Declaration of Helsinki.

Results

Basic clinical parameters of ACS patients are shown in Table II. The results of gene expression are shown as studied gene to β-actin mRNA level ratio.

Compared to healthy subjects, ACS patients showed slightly elevated transcriptional activity of genes encoding TGFβ1 (0.29 ± 0.043 vs. 0.08 ± 0.020 , $p < 0.05$) and its receptors RI and RII (0.071 ± 0.022 vs. 0.036 ± 0.023 , $p < 0.05$; 0.134 ± 0.020 vs. 0.048 ± 0.016 , $p < 0.05$, respectively). After 5 days of standard treatment a statistically significant decrease in TGFβRI gene expression was observed (0.071 ± 0.022 vs. 0.043 ± 0.021 , $p < 0.01$) while other studied genes' mRNA level remained similar during the observation period (Fig. 2). Analysis of ACS subgroups revealed that there are no significant

differences in expression of studied genes at day 1 (although UA tended to have higher TGFβRI expression). After 5 days a statistically significant decrease was observed only for the TGFβRI gene in the UA group (Table IV).

Additionally, the RI/RII gene expression ratio was evaluated. On the day of ACS, the RI/RII ratio was higher in the study group than in controls (0.75 ± 0.218 vs. 0.42 ± 0.202 , $p < 0.05$), but after 5-day therapy it decreased significantly (0.75 ± 0.218 vs. 0.29 ± 0.065 , $p < 0.05$) and was lower than in controls (0.29 ± 0.065 vs. 0.42 ± 0.202 , $p < 0.05$). Again, the decrease was more pronounced in UA than in MI subgroups (Table IV).

mRNA level of TGFβ1 did not correlate with either of the receptors. The expression of genes encoding receptors were not correlated either. Studied gene expression did not differ among ACS subgroups.

No major atherosclerosis risk factors (obesity, smoking, dyslipidaemia, hypertension, age/sex) affected expression of the studied genes. The exception was the TGFβRI gene, whose expression was higher in ACS patients with family history of CAD (0.34 ± 0.159 vs. 0.11 ± 0.04 ; $p < 0.05$). Moreover, the presence of 4 or more classical risk factors correlated with higher level of TGFβRI expression (0.098 ± 0.035 vs. 0.028 ± 0.008 ; $p < 0.05$). hsCRP did not correlate significantly either with TGFβ ($r = 0.4$; $p = \text{NS}$) or with RI and RII receptors ($r = 0.2$; $p = \text{NS}$ and $r = -0.1$; $p = \text{NS}$ respectively).

The analysis of the association between studied gene expression and such clinical variables as ejection fraction, myocardial necrosis markers (CK, CKMB, Tnl), time to PCI and early risk score assessed by the GRACE model and GFR did not reveal any statistically significant relations. Angiographic findings (e.g. number of affected vessels and ACS-related artery) were not related to studied genes' mRNA levels either.

Discussion

The effect of TGFβ on the vascular system has been proven to be protective. According to Grainger's hypothesis, lack of TGFβ activity leads to a two-step process: If the activity is transiently diminished, an atherosclerotic lesion is formed, but since TGFβ still stimulates ECM production, it results in a stable plaque phenotype. If TGFβ activity is chronically reduced, inflammatory processes may lead to the plaque being converted to an unstable phenotype. The data come mostly from animal models of atherosclerosis. The observations in humans, however, particularly those assessing plasma levels of TGFβ and their correlation with clinical manifestation of atherosclerosis, remain confusing.

Although Grainger *et al.* in 1995 published their data proving that patients with atherosclerosis had

Table IV. Gene expression of TGF- and its receptors in ACS subgroups

| Gene expression | ACS subgroups | | |
|---------------------|----------------|----------------|--------------|
| | STEMI (n = 14) | NSTEMI (n = 9) | UA (n = 9) |
| TGFβ-1 (day 1) | 0.29 ±0.05 | 0.23 ±0.05 | 0.34 ±0.12 |
| TGFβ-1 (day 5) | 0.33 ±0.06 | 0.26 ±0.05 | 0.26 ±0.04 |
| TGFβRI (day 1) | 0.04 ±0.01 | 0.03 ±0.01 | 0.15 ±0.07 |
| TGFβRI (day 5) | 0.07 ±0.05 | 0.03 ±0.004 | 0.02 ±0.004* |
| TGFβRII (day 1) | 0.18 ±0.04 | 0.10 ±0.02 | 0.10 ±0.02 |
| TGFβRII (day 5) | 0.11 ±0.04 | 0.24 ±0.10 | 0.10 ±0.02 |
| TGFβ RI/RII (day 1) | 0.35 ±0.07 | 0.56 ±0.30 | 1.47 ±0.61 |
| TGFβ RI/RII (day 5) | 0.40 ±0.16 | 0.22 ±0.06 | 0.24 ±0.05* |

* $p < 0.05$ statistically significant difference vs. day 1 values

severely depressed circulating levels of TGFβ [9], some later reports agreed with the findings [10], while some others did not find any correlation of TGFβ and atherosclerosis [11] or even suggested that atherosclerotic patients have increased TGFβ levels [12,13]. The discrepancies are mostly due to methodology: some investigators assessed total cytokine, while some others assessed active cytokine. It is noteworthy that investigators who observed low levels of active TGFβ also noted similar or even elevated concentrations of total TGFβ in the atherosclerotic population compared to healthy controls. The reason for low levels of active cytokine in CAD subjects might be that in this group of patients high levels of TGFβ inhibitors (e.g. lipoprotein (a), plasminogen activator inhibitor type 1) are observed [14, 15].

Recently the emphasis has been on leukocytes as a potential source of TGFβ. It is not clear, however, which population is the main source. In this study we have observed elevated transcriptional activity of genes encoding TGFβ1 and its two receptors in circulating monocytes of ACS patients. If blood monocytes are to be considered an important source of circulating cytokines, our finding would confirm the data that in atherosclerosis TGFβ production is not diminished or can even be increased. On the other hand, recent findings of Kempf *et al.* [16] as well as our previous report [17] show that TGFβ mRNA expression in PBMC is lower in CAD subjects. The reason might be that PBMC are a heterogeneous group of cells (e.g. a high proportion of lymphocytes) and our new data seem to provide more detailed information. Our study showed increased gene expression of TGFβ in all clinical forms of ACS with no differences among ACS subgroups.

Besides TGFβ itself, changes in TGFβ receptor signalling play an important role in the evolution of atherosclerotic lesions. In the normal vessel wall,

expression of TGF β type II receptor predominates, while in the pathological condition TGF β RI signalling increases. In the present study transcriptional activity of TGF β receptor genes was elevated along with the cytokine gene. The RI/RII expression ratio was the highest on the first day of ACS, which means that the balance was more toward the "pathological" RI receptor. 5-day therapy did not affect either TGF β 1 gene expression or RII expression, but reduced RI, thus restoring the balance between 'pathological' and 'physiological' receptor. This finding is important considering that receptor disturbances in TGF β signalling may have a profound effect on atherosclerosis progression [18]. It is noteworthy, however, that the decrease in TGF β RI expression and RI/RII ratio was significantly pronounced only in the UA group. It is possible that the overlap of necrosis-related inflammatory reaction in STEMI and NSTEMI subgroups had some effect on TGF β R expression on day 5, but the design of the study does not allow us to address this issue.

The day 1 sample is considered in the study as prior to treatment status. As mentioned, before the collection patients could only receive emergency ACS treatment, so the treatment given prior to sampling is unlikely to have had any effect on gene expression. Five days of treatment did not affect the monocyte expression of the TGF β and RII gene. Although there are a number of articles reporting the effect of statins, ACE inhibitors and aspirin on TGF β [19, 20], multi-drug therapy varying from patient to patient and the short observation time did not allow us to observe any major effect on the studied genes.

TGF β seems to be an independent mechanism in atherogenesis and it is not surprising that in the study we have not observed any linkage between gene expression of TGF β or its receptors and classical CAD risk factors, such as hsCRP level, smoking, dyslipidaemia, hypertension and obesity. There are some reports, however, to confirm the mechanistic link between cigarette smoking or dyslipidaemia and TGF β [21, 22]. Although no correlation was found between studied genes and particular risk factors, patients with a higher total risk score (e.g. more than 3 risk factors) presented with elevated level of TGF β RI gene expression, which is further proof that the balance in the receptors is important in maintaining proper TGF β signalling and its protective action. In the subsequent analysis patients with a family history of CAD had a higher gene expression of "pathological" TGF β RI receptor and, consequently, increased RI/RII ratio. The gene of TGF β itself did not differentiate the groups. There are no data in the literature to prove that molecular disturbances in TGF β signalling might be one of the genetic backgrounds of atherosclerosis, and this is a novel

finding in this study. A few polymorphisms have been identified to link genetic background and ACS, but they all referred to the TGF β gene, not its receptors [23].

Lack of a correlation between studied gene expression and clinical parameters of ACS (including EF, time of chest pain before PCI, necrosis markers) is not surprising given the fact that monocyte gene expression is more likely to reflect a chronic inflammatory process and monocyte activation leading to ACS than the clinical state at the time of the event. Also renal function (eGFR), which is strongly associated with cardiovascular risk (24), due to exclusion criteria was homogeneous in the studied group and did not correlate with monocyte gene expression.

One limitation of the study is the number of patients recruited. An increased number would possibly reveal some relationships that did not meet statistical significance here. The exclusion criteria, however, were set to keep study subgroups relatively homogeneous. Another issue is that the study protocol does not allow us to distinguish whether studied gene expression changes were due to the chronic atherosclerotic process or acute plaque rupture. Although a stable CAD group would provide some more information on this matter, it is difficult to obtain a relatively homogeneous stable angina group with similar treatment, which, presumably, is an important factor affecting gene expression.

The altered gene expression observed here in monocytes of ACS patients reflects a systemic reaction to atherosclerosis, and the part TGF β takes in it. It would be of interest to evaluate it in lymphocytes as well, and to correlate these findings with circulating levels of TGF β .

In conclusion, monocytes of ACS patients demonstrate overexpression of TGF β 1 and its receptors' genes, which suggests activation of protective mechanisms in ACS. Increased TGF β RI expression in high risk patients may reflect the significance of this receptor in pathology of atherosclerosis. 5-day standard treatment downregulated the TGF β RI gene but did not affect TGF β 1 and TGF β RII.

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