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Contents of mRNAs Encoding Endosome/Lysosome Components in Normal Human Aorta and in Stage II of Atherogenesis: a Hidden Regulation

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Abstract—Contents of mRNAs encoding endosome/lysosome components EEA1, Rab5a, Lamp1, Lamp2, p62 (SQSTM1), and CD63 were measured by quantitative PCR and compared in intact fragments of human aorta and in aorta fragments with atherosclerotic lesions of stage II (fatty streaks) of the same donors. During atherogenesis an increase was detected only in the level of p62 mRNA but not in other mRNAs. Nevertheless, correlation analysis revealed a profound rearrangement of inter-gene correlations: only 30% of correlations found in the fatty streaks coincided with the correlations in normal fragments. Thus, new constellations were formed in fatty streaks concurrently with disappearance of correlations between mRNAs under study and mRNAs encoding factors of lipid accumulation, reverse cholesterol transfer, and some lipid sensors/transcription regulators of lipid metabolism.

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Key words: endosomes, lysosomes, autophagy, atherogenesis, PCR, gene expression

Atherogenesis is characterized by lipid accumulation in intimal cells. This accumulation is different from the normal accumulation by the mechanism of capture by the cells of lipoprotein lipids, by the intracellular location of lipid stores, and by the course of lipid mobilization [1, 2]. We supposed that in atherogenesis the system of inner translocation and storage of lipids with involvement of endosomes-(phagosomes)-lysosomes could be activated not only on the level of interactions between protein components of these compartments (as already demonstrated in model systems on the cell loading with modified lipoproteins [3]), but also on the level of expression of the corresponding genes. As markers of these compartments we used the early endosome antigen 1 (EEA1) and interacting with it a small GTPase, Rab5a (responsible for translocation and fusion of early endosomes [4, 5]),

Abbreviations: ABCA1 and ABCG1, ATP-binding cassette transporters A1 and G1; ACAT1, acyl-CoA-cholesterol acyltransferase 1; ApoE, apolipoprotein E; AR, androgen receptor; Aro, aromatase; CCL18, C–C motif-containing chemokine 18; CD36, CD63, and CD68, differentiation clusters 36, 63, and 68; CEH, cholesteryl ester neutral hydrolase; EEA1, early endosome antigen 1; ERα, estrogen receptor α; E-Sel, E-selectin; EST, estrogen sulfotransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ICAM1, intercellular adhesion molecule 1; Lamp 1/2, lysosome-associated membrane glycoproteins 1 and 2; LDLR, low density lipoprotein receptor; LPL, lipoprotein lipase; LXRα and LXRβ, liver X receptors α and β; p62, 62-kDa protein; PPARα and PPARγ, peroxisome proliferator-activated receptors α and γ; SR-A and SR-BI, scavenger receptors A and BI; SREBP1 and SREBP2, sterol regulatory element-binding proteins 1 and 2; STS, steroid sulfatase; TfR1, transferrin receptor 1; TLR4, Toll-like receptor 4; TNFα, tumor necrosis factor α; VCAM1, vascular cell adhesion molecule 1.

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lysosome-associated membrane glycoproteins 1 and 2 (Lamp 1 and 2) (responsible for biogenesis of lysosomes, translocation and fusion of phagosomes with lysosomes [6]), protein p62 (responsible for delivery of ubiquitinylated proteins into autophagosomes [7]), and antigen CD63 (responsible for exocytosis and possibly for biogenesis of lysosomes [8]). Lamp1, Lamp2, p62, and CD63 can displace between the plasma membrane, late endosomes, and lysosomes, and thus do not belong to the same compartment. Determination of contents of mRNAs of these markers in intact specimens and specimens with stage II of atherosclerotic damage (fatty streak) taken from the same donors showed a significant change (an increase) only in one parameter (p62). However, analysis of correlations between mRNAs revealed a significant rearrangement in the regulatory system of expression of the corresponding genes during the early stage of atherogenesis.

MATERIALS AND METHODS

In the present work seven pairs (normal state – pathology) of human aorta fragments obtained at autopsy from six men and one woman 31-54-years-old were studied. The aorta fragments were dissected longitudinally and washed with isotonic phosphate buffer; the intima from apparently normal and from atherosclerotically changed regions were separated mechanically, frozen in liquid nitrogen, and stored at –70°C.

The methods of analysis have been described in detail in [9]. RNAs were isolated from the frozen specimens using a TRIzol Reagent (Invitrogen, USA). The cDNA was synthesized on total RNA using the ImProm-II™ Reverse Transcription System (Promega, USA). The resulting cDNA was used as a template for PCR in a real time regimen using a Rotor-Gene 3000 amplifier (Corbett Research, Australia) equipped with reagents including an intercalating dye SYBR Green I (Syntol, Russia) according to the description attached to the reagent set. Primers were selected using the Beacon Designer 6.00 Program (www.PremierBiosoft.com). To confirm the expected sequence, the amplification products were sequenced using the ABI PRISM® BigDye™ Terminator v.3.1 reagent set and an ABI PRISM 3100-Avant automated DNA sequencer. Only those PCR results were taken into account when the melting temperature of amplification products and their electrophoretic mobility corresponded to the expected values. The primer sequences are presented in Table 1. Contents of individual mRNAs in the studied samples are expressed in percent of the content of mRNA of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as an internal reference control.

The results were processed statistically using the Statistica 7.0 Program Package. The significance of differences between the values was assessed using the Mann–Whitney test. Correlations were assessed according to Spearman rank correlation. Differences or correlations were considered significant at $p < 0.05$. Mean values and standard deviations are presented.

RESULTS AND DISCUSSION

The mRNAs encoding the studied endosome/lysosome components were prominently expressed in intact fragments of the aorta, and the level of their expression was equal or higher than that of mRNA of the housekeeping gene of GAPDH (Fig. 1a). At stage II of atherogenesis (fatty streak) a significant change (an increase) was recorded only for mRNA of p62 (Fig. 1b).

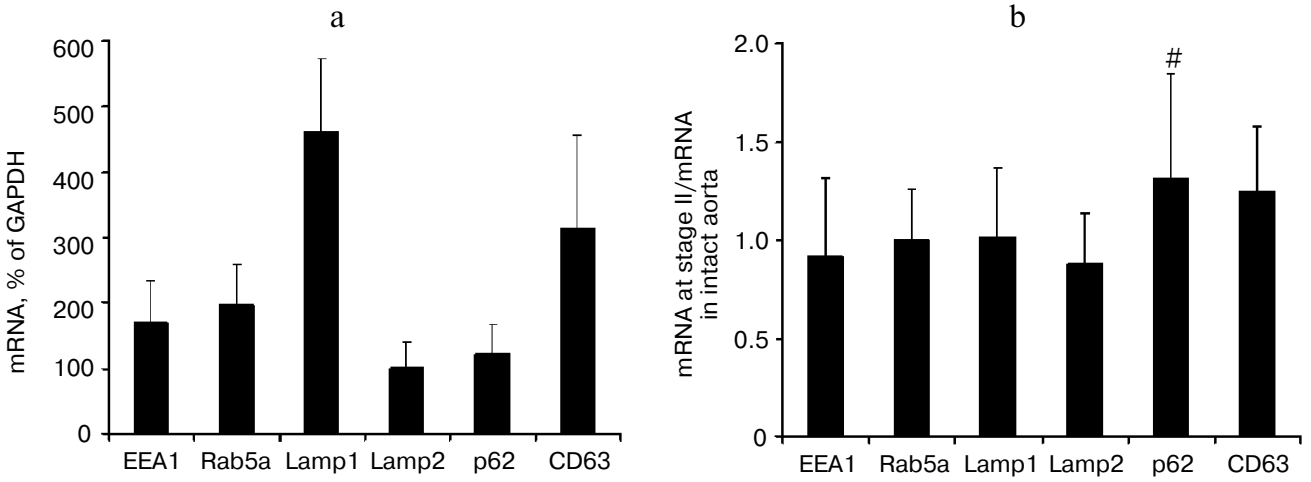

Fig. 1. GAPDH-normalized contents of mRNAs of lysosome/endosome components in intact fragments of human aorta (a) and the ratio between the contents of these mRNAs in aorta fragments with atherosclerotic damage (stage II) and in intact fragments of the aorta from the same donors (b). Mean values ± standard deviations are presented. The symbol # indicates a significant deviation from unity.

Table 1. Primers used for measurements of mRNAs

mRNA	Sequence	Size of PCR product, bp
1	2	3
GAPDH	for 5'-GAGCCCGCAGCCTCCCGCT-3' rev 5'-GCGCCCAATACGACCAAATC-3'	145
EEA1	for 5'-GGAGGAGAGTCTAATCTTGCTTTG-3' rev 5'-GAATCAGTCACCAACCCATCAG-3'	182
Rab5a	for 5'-CAGTTCAAACTAGTACTTCTGG-3' rev 5'-GCTAGGCTATGGTATCGTTCTTG-3'	200
Lamp1	for 5'-AACTTCTCTGCTGCCTTCTC-3' rev 5'-GAGTGAGTGTATGTCCTCTTCC-3'	172
Lamp2	for 5'-GATACTTGTCTGCTGGCTACC-3' rev 5'-CATGCTGATGTTCACTTCCTTC-3	222
p62 lck	for 5'-CCGAGTGTGAATTTCCTGAAG-3' rev 5'-CTCTGTGCTGGAACTCTCTG-3'	144
CD63	for 5'-GTGTGAAGTTCTTGCTCTACG-3' rev 5'-ACTGCGATGATGACCACTG-3'	154
STS	for 5'-ACTGCAACGCCTACTTAAATG-3' rev 5'-AGGGTCTGGGTGTGTCTGTC-3'	289
Arom	for 5'-GTGAAAAAGGGGACAAACAT-3' rev 5'-TGGAATCGTCTCAGAAGTGT-3'	215
EST	for 5'-TTGGTGGGAAAAGGGAAAGAGTC-3' rev 5'-CTGGCAGTGTTGTGTAATTTGTGG-3'	200
AR	for 5'-CCTGGCTTCCGCAACTTACAC-3' rev 5'-GGACTTGTGCATGCGGTACTCA-3'	168
ERα	for 5'-CTGGCTACATCATCTCGGTTC-3' rev 5'-AGGTGGATCAAAGTGTCTGTG-3'	182
TfR1	for 5'-GTTGAATTGAACCTGGACTATGAG-3' rev 5'-GTCTGGAAGTAGCACGGAAG-3'	154
E-sel	for 5'-CAGGTGAACCCAACAATAGGC-3' rev 5'-CCAGGCTTCCATGCTCAGG-3'	279
ICAM1	for 5'-AGGTGTATGAACTGAGCAATGTG-3' rev 5'-CTGGCAGCGTAGGGTAAGG-3'	179
VCAM1	for 5'-AGCGGAGACAGGAGACACAG-3' rev 5'-AATGGCAGGTATTATTAAGGAGGATG-3'	226
LDLR	for 5'-ACGGTGGAGATAGTGACAATG-3' rev 5'-GTCCTGGTTGTGGCAAATGTG-3'	249
LPL	for 5'-TCAATCACAGCAGCAAAACCTTC-3' rev 5'-ACAGCCAGTCCACCACAATG-3'	138

Table 1 (Contd.)

1	2	3
SR-A	for 5'-GCCCTTTACCTCCTCGTGTTTG-3' rev 5'-CATTTCCTCTTCGCTGTCATTTCC-3'	162
CD36	for 5'-GGTGCTGTCCTGGCTGTG-3' rev 5'-GCTTAACTTGAATGTTGCTGCTG-3'	214
CD68	for 5'-ATTCATGCAGGACCTCCAGC-3' rev 5'-AGGAGAAACTTTGCCCAAAG-3'	263
TLR4	for 5'-TTCTACAAAATCCCCGACAAC-3' rev 5'-GAGGTGGCTTAGGCTCTG-3'	180
ACAT1	for 5'-CCTACCCTTATCTACCGTGACAG -3' rev 5'-AGCACACCTGGCAAGATGG -3'	215
ABCA1	for 5'-CTGAAGCCAATCCTGAGAACAC-3' rev 5'-CCGCAGACAATACGAGACAC-3'	224
ABCG1	for 5'-GAGACGGACCTGCTGAATGG-3' rev 5'-CCAACTCACCACTATTGAACTTCC-3'	208
CEH	for 5'-AACCACCACCTCTGCTGTC-3' rev 5'-CCAAACTCCTGCTTGTTAATTCC-3'	243
ApoE	for 5'-GGGTCGCTTTTGGGATTAC-3' rev 5'-GTCAGTTGTTCCTCCAGTTC-3'	159
SR-BI	for 5'-CCCTTGTTTCTCTCCCATCCTC-3' rev 5'-AATCTTCCCAGTTTGTCCAATGC-3'	204
LXRα	for 5'-GTACAACCCTGGGAGTGAGAG-3' rev 5'-TGATAGCAATGAGCAAGGCAAAC-3'	173
LXRβ	for 5'-CACGAGACAGAGTGTATCACCTTC-3' rev 5'-AGATGTTGATGGCGATGAGCAG-3'	172
PPARα	for 5'-GGCGAGGATAGTTCTGGAAGC-3' rev 5'-CACAGGATAAGTCACCGAGGAG-3'	132
PPARγ	for 5'-TGTCGGTTTCAGAAATGCCTTG-3' rev 5'-CTCGCCTTTGCTTTGGTCAG-3'	209
SREBP1	for 5'-CTGAGGCAAAGCTGAATAAATCT-3' rev 5'-GCCGACACCAGATCCTTCA-3'	151
SREBP2	for 5'-TCACCACCCCTATCCAGACG-3' rev 5'-CCTTCCTCAGAACGCCAGAC-3'	288
TNFα	for 5'-CTTCTCCTTCCTGATCGTGG-3' rev 5'-AGGGTTTGCTACAACATGGG-3'	184
CCL18	for 5'-CCTCCTTGTCCTCGTCTG-3' rev 5'-GGGCTGGTTTCAGAATAGTC-3'	135

Table 2. Correlations between contents of mRNAs of endosome/lysosome components and some other mRNAs in intact fragments of human aorta and in aorta fragments with atherosclerotic damage. Significant positive and negative correlations are shown by black and gray color, respectively; dots indicate correlations that are common for the normal state and pathology

Functional cluster	mRNA	Intact aorta						Fatty streak					
		EEA1	Rab5a	Lamp1	Lamp2	p62	CD63	EEA1	Rab5a	Lamp1	Lamp2	p62	CD63
Metabolism/ action of sex hormones	STS												
	Aro												
	EST												
	AR	●						●					
	ERα					●						●	
Cell adhesion	E-Sel												
	ICAM1												
	VCAM1												
Lipid capture/ accumulation	LDLR												
	LPL												
	SR-A												
	CD36												
	CD68												
	TLR4												
	ACAT1												
Reverse cholesterol transport	ABCA1												
	ABCG1												
	CEH												
	ApoE												
	SR-BI												
Lipid sensors/ transcriptional regulators of lipid metabolism	LXRα												
	LXRβ					●						●	
	PPARα		●						●				
	PPARγ												
	SREBP1												
	SREBP2		●						●				
Inflammation/ repair factors	TNFα												
	CCL18		●						●				
Endosome/ lysosome components	EEA1												
	Rab5a					●						●	
	Lamp1												
	Lamp2												
	p62												

Correlations were analyzed between the contents of these and other mRNAs in the aorta intact fragments and in those with atherosclerotic damage (Table 2). It was shown that in atherogenesis the system of expression coupling was significantly changed not only within the group of mRNAs encoding endosome/lysosome components, but also between mRNAs of this group and other mRNAs. Only seven of 23 (30%) correlations revealed in the fatty streak were coincident with correlations detected in the normal tissue.

The character of correlations presented in Table 2 for the intact aorta allowed us to subdivide mRNAs encoding endosome/lysosome components into two subgroups: the first subgroup included EEA1 and Lamp1, and the second subgroup included Rab5a, Lamp2, p62, and CD63. Except for the correlation of EEA1 mRNA with mRNA of the androgen receptor (AR), the first subgroup members did not correlate with other mRNAs studied. The second subgroup members had multiple correlations both inside the subgroup and with other mRNAs. The resemblance in the distribution of correlations was especially pronounced between mRNAs of Lamp 2 and CD63: seven of nine correlations of each were common. The finding of mRNAs of EEA1 and Rab5a in different regulatory constellations seems rather surprising because these two proteins are functioning in close cooperation [10]. In atherogenesis correlations between mRNAs were significantly changed: mRNA of EEA1 began to correlate with mRNAs of the second subgroup (including mRNA of Rab5a) and was involved in correlations with other types of mRNAs. However, mRNA of Lamp1 remained without correlations with other mRNAs. Comparison of the right and left parts of Table 2 indicates the most pronounced differences between the normal state and pathology including: full (from 13 to 0) disappearance of correlations between mRNAs of the endosome/lysosome components and of lipid accumulation factors in cells (CD36, CD68, TLR4, ACAT1) and factors of reverse cholesterol transport from cells (ABCA1, SR-BI); disappearance of correlations with mRNA of the fatty acid sensor PPARγ; appearance of a significant number of negative correlations. A small number of correlations in the fatty streak coinciding with correlations in the intact tissue suggested that under conditions of pathology correlations should dominate that could not be detected in normal tissue. The most conservative were correlations formed by mRNAs of Rab5a and p62. A significant rearrangement of the regulatory system was also revealed on analyzing correlations between contents of mRNA in the intact and damaged fragments of the aorta: correlation was detected only for mRNA of Lamp2. Figure 2 demonstrates changes in the profile of correlations for mRNA of each endosome/lysosome component on transition from the normal state to pathology.

Changes in the number of correlations were especially alike in the case of mRNAs of Lamp2 and CD63 (full disappearance of positive correlations and appearance of negative ones). Thus, the similarity of regulation of these two mRNAs was obvious not only in the normal state but also in pathology.

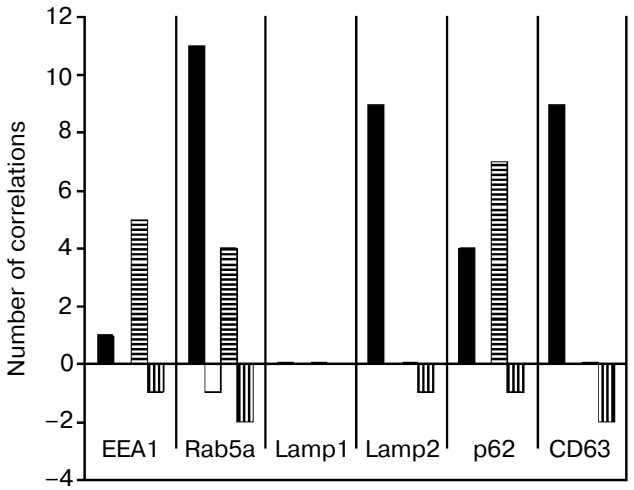

Fig. 2. Profiles of correlations between contents of mRNAs encoding endosome/lysosome components and contents of other mRNAs. Dark and white columns present, respectively, positive and negative correlations in the normal state, columns with horizontal and vertical hatching present, respectively, positive and negative correlations in the fatty streak.

Expression of the proteins measured in the present work on the level of mRNAs was virtually unstudied in atherogenesis. It was found [11] that in atherosclerosis-prone rabbits of WHHR strain the content of mRNA of CD63 in the aorta increased with age. In experiments with macrophage cultures, p62 was shown to be induced on the level of protein and mRNA under conditions of oxidative stress and under the influence of bacterial lipopolysaccharides, and this induction included the interaction of the activated transcription factor NRF2 with the antioxidant-responsive element of the gene *p62* promoter [12, 13]. In the fatty streak macrophages are an insignificant fraction of the cells (from 0 to 4% by data of immunohistochemical analysis [14]), thus the increase in the p62 mRNA found by us seems to be associated with an increase in its expression in cells other than macrophages, and it was reasonable to suggests that this increase could activate autophagy in these cells in atherogenesis. Autophagy is a pathway of lipid elimination in the cell [15], and thus the increase in the level of p62 mRNA in the fatty streak can contribute to homeostasis under conditions of lipid excess. Earlier an increase in the Lamp2 on the protein level was recorded on incubation of macrophages with aggregated low density lipoproteins [16]. The absence of even a tendency for increase in the level of Lamp2 mRNA in the fatty streak as compared to the intact intima of the aorta in our samples suggests that regulatory mechanisms of expression of this protein in

macrophages and other cells of vessels could be different. A similar hypothesis seems to be reasonable about the regulation of EEA1 and Rab5a expression: the contents of these components of early endosomes increased in macrophages on the protein level under the influence of endocytosis inducers (IL4 and PGE2) [17], whereas the contents of their mRNAs in the fatty streak did not change relative to the normal state.

Detecting correlations between mRNAs, those which change on transition from the normal state to pathology and conservative ones, can serve as a good guide for identification of transcription factors that really control gene expression under different conditions. Thus, based on the presented data it can be rather reliably predicted that the regulatory sequences of Lamp2 and CD63 should have similar enhancer elements interacting with the same transcription factor that is active in the normal tissue and loses its activity in atherogenesis. Detecting a number of conservative correlations for mRNAs of Rab5a and p62, including the correlations between these mRNAs as they are, indicate that the regulatory regions of these two genes should contain similar enhancer elements controlled by a common constitutively active transcription factor, and that such element should be absent in the genes whose mRNAs lose the correlation with mRNAs of Rab5a and p62 in atherogenesis. In this connection, note that four of six conservative correlations of mRNAs of Rab5a and p62 are represented by mRNAs of the transcription factors (LXRβ, PPARα, SREBP2, and ERα).

The findings suggest that in the course of atherogenesis the system of regulation of vesicular transport in aorta intima cells on the level of mRNAs loses a significant fraction of normal correlations with the factors of entrance, elimination, and autoregulation of lipids. It may be that this regulation, hidden due to absence of obvious changes in the mRNA level, can contribute to formation of foam cells.

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