1166-9 Multi-Functions of Endogenous Opioids on the Process of Coronary Arteriosclerosis

K. Iwai, M. Matsumoto, Y.-B. Deng, J. Kato, J. Tanouchi, Y. Yamada. *Kanazawa Medical University, Ishikawa, Japan*

Background: Recently, the endogenous opioids are proved to be the chemical mediators of chronic inflammation in the peripheral tissues. For the first time, we investigated their roles in the process of coronary arteriosclerosis.

Methods: The distributions of β-endorphin (END), met-enkephalin (ENK) and opioid receptors (subtypes: μ, δ, κ) were investigated by immunohistochemistry and in situ hybridization (digoxygenin-labeled PCR probes) in the human coronary arteries obtained by directional atherectomy (angina pectoris (n = 45) and acute myocardial infarction (9)) and by autopsy (10). Moreover, their biological effects on the smooth muscle cells were investigated by the in vitro cultured system.

Results: The immunoreactivities (IR) of END and ENK were observed in the autonomic nerve fibers in the adventitia, the smooth muscle cells (SMC) both in the neointima and media. In the plaques, these IR showed high intensities on the infiltrating macrophages and T-lymphocytes and on some demarcated areas of the intermittium. The signals of mRNA for END and ENK and the IR of the opioid receptors were detected in all these cells, indicating that they produced and received the opioids. Moreover, the proliferating activities (cell number) of SMC in vitro was inhibited approximately 10% by END or ENK (10 pg/ml), which was restored by pretreatment with phorbolesters. The gene expression of the matrix metalloproteinase-9 of SMC was significantly up-regulated by END or ENK (RT-PCR). These results suggest that opioids may actually influence on the SMC in vivo, leading to aggravate the coronary arteriosclerosis.

Conclusion: The opioids were produced, released and received by autocrine and paracrine mechanisms among nerve end, smooth muscle cells and inflammatory cells. Opioids are the mediators of smooth muscle cells to cross talk with neuro-immuno syatems in the inflammation processes of coronary arteriosclerosis.

1166-10 Expression of Adhesion Molecules on Neovessels in Atherosclerotic Plaques: Differences Between Lipid-rich and Fibrous Plaques

O.J. de Boer, A.C. van der Wal, A.E. Becker. *Dept of Cardiovascular Pathology, Academic Medical Center, the Netherlands*

Background: Studies on the role of adhesion molecules in atherosclerosis focus on luminal endothelial cells. However, it is an old observation that neovessels are present in the intima of advanced atherosclerotic plaques and in this context the importance of these vessels neglected.

Methods: In the present study we analyzed the presence of neovessels, and their expression of adhesion molecules in lipid rich and fibrous plaques. The presence of neovessels and their expression of adhesion molecules was correlated with the presence of inflammatory cells (lymphocytes and macrophages). ICAM-1, VCAM-1, E-selectin and CD40, recently found to be expressed by activated endothelial cells, and it's ligand, CD40L were studied.

Results: Presence of neovessels, relation to inflammation and expression of adhesion molecules is summarized in the table.

Type of plaque	neo-vessels present	vessels/ mm2	inflam-mation	VCAM-1*	E-selectin*	CD40*
Fibrous (n = 10)	4/10	0.6 ± 0.7		1%	4%	1%
Lipid rich (n = 15)	14/15	2.1 ± 2.6	++	many**	41%	27%

* *percentage of vessels positive;* ** *not countable because of extensive overall positivity of cells*

The expression of CD40 was not related to that of any of the other adhesion molecules. CD40L was found to be expressed by mononuclear cells, especially lymphocytes, usually subendothelially.

Conclusions: Our results indicate that neovessels may be an important gateway for the migration of immunocompetent cells into advanced lipid rich plaques. Interactions between CD40+ endothelial cells and CD40L+ lymphocytes may play a regulatory role in this process.

1166-11 The Prevalence of Inflammation of Plaques in the Coronary Artery Without Plaque Rupture

G. Pasterkamp, D.J. Hijnen, A.S. Schoneveld, S. Plomp, B. Hillen, A.E. van der Wal, H.L. Teepen, C. Borst *Departments of Functional Anatomy and Cardiology, Utrecht University Hospital, Utrecht, The Netherlands*

Background: Retrospectively, plaque rupture is often co-localized with inflammation of the cap and shoulder of the atherosclerotic plaque. Previously, we showed that inflammation is frequent in unruptured plaques of femoral arteries. The prevalence of inflammation in plaques of the atherosclerotic coronary artery is unknown.

Methods: Cross-sections (n = 240) were obtained from 62 atherosclerotic coronary arteries and stained on CD68 or alkalin phosphatase (macrophages) (n = 240). Cross-sections obtained from 29 coronary arteries were also stained for the presence of CD45RO (T-lymphocytes) (n = 130). The degree of staining in the cap and shoulder of the plaque was expressed as being absent or minor versus moderately or heavily positive.

Results: The cap of the plaque revealed moderately or heavily positive staining for macrophages and T-lymphocytes in 30% and 24% of the cross-sections, respectively. The shoulder of the plaque revealed moderately or heavily positive staining for macrophages or T-lymphocytes in 26% and 15% of the cross-sections, respectively. In the cap of the plaque, positive staining for macrophages or T-lymphocytes was observed in at least one cross-section in 38/62 and 15/29 of the arteries, respectively. In the shoulder of the plaque, positive staining for macrophages and T-lymphocytes was observed in at least one cross-section in 40/62 and 12/29 of the arteries, respectively.

Conclusion: Inflammation of the cap and shoulder of the plaque is a common feauture in the coronary atherosclerotic artery.

1166-12 Expression and Regulation of a Novel Collagen-binding Heat Shock Protein in Vascular Lesion Formation

E.F. Rocnik, J.G. Pickering. *University of Western Ontario; John P. Robarts Research Institute, London, Canada*

Background: Colligin is a recently discovered 46 kD heat shock protein that binds collagen and may act as a molecular chaperone. Evidence is emerging that chaperones are critical to protein folding and secretion in health and disease. There is no data, however, on collagen chaperones in vascular pathobiology.

Methods: To determine if colligin was expressed during neointimal formation, the left carotid artery of rats was injured and assessed by immunhistochemistry. To assess regulation of colligin expression, SMCs were freshly dispersed from porcine carotid arteries and expression of colligin and type I collagen was tracked by Western blot analysis as the cells dedifferentiated in culture. The effect of FGF-2 and phorbol ester on both colligin and collagen was assessed by Northern and Western blot analysis.

Results: In situ, colligin was detected in adventitial fibroblasts but only faintly detected in SMCs of the normal rat carotid artery. However, at 4 and 14 days after injury intense colligin expression was evident in SMCs of the inner media and the developing neointima. In vitro, little colligin was detected in freshly dispersed cells but expression significantly increased over the first 5 days in culture. This profile closely matched that of proα1 (I) collagen. FGF-2 produced a dose-dependent decrease in both colligin and proα1 (I) collagen protein expression, with reductions of mRNA to 0.4 and 0.1 of basal levels, respectively. Treatment (10 h) with phorbol myristate acetate decreased both colligin and proα1 (I) collagen mRNA to 0.4 and 0.3 of basal levels, respectively.

Conclusions: Colligin is a newly recognized heat shock protein that is expressed by SMCs during neointimal formation and is tightly coregulated with collagen under diverse stimuli. This novel chaperone may thus be critical to maintaining the fidelity of collagen elaboration by SMCs and thereby influence lesion formation and plaque stability.

1166-13 Proliferation in Different Stages of Development of Atherosclerotic Lesions in APOE*3-Leiden Mice

E. Lutgens[1], E.D. de Muinck[1], P.A.F.M. Doevendans[1], L.M. Havekes[2], H.J.J. Wellens[1], M.J.A.P. Daemen[2]. [1]*Department of Cardiology, Cardiovascular Research Institute Maastricht, and TNO-PG Leiden, the Netherlands;* [2]*Department of Pathology, Cardiovascular Research Institute Maastricht, and TNO-PG Leiden, the Netherlands*

Because cell proliferation is thought to be of importance in atherogenesis, we determined the amount of proliferation in atherosclerotic plaques in APOE*3-Leiden mice that express a defective human apo-E gene. APOE*3-Leiden mice (n = 6) were fed a high fat/cholesterol diet for 6 months. To label DNA-synthesizing cells, 5'-bromo-2'-deoxyuridine (BrdU) was given 1 week before. In total 135 lesions (type 1–5, AHA classification) were present throughout the arterial tree. In all plaque types, the percentage BrdU-positive nuclei was increased as compared to the non-diseased arterial wall (control). The highest labeling-index (LI) was found in initial type 2 lesions (8.6 ± 0.8% vs 1.0 ± 0.2% in controls). BrdU labeling decreased to 5.8 ± 0.7% in complex type 5 lesions. The pattern and level of BrdU labeling changed with progression of the lesion. Medial labeling decreased from 8.6 ± 0.8% in type 2 lesions to 0.7 ± 0.2% in type 5 lesions. In type 3 and 4 lesions, labeling shifted to the intimal shoulder region (2.3 ± 0.8% and 3.2 ± 0.3% respectively) and to endothelial cells covering the lesion (2.1 ± 0.8% and 3.2 ± 0.3% respectively). In type 5 lesions, the majority of the labeling was present in the shoulder region (2.1 ± 0.3%), whereas the LI

had decreased to 0.7 ± 0.2% in endothelial cells covering the lesion. The fibrous cap of type 5 lesions showed a LI of 1.7 ± 0.3%. These data show that in APOE*3-Leiden mice, the highest level of proliferation occurs in type 2 lesions. However, when the lesions progress, proliferation decreases. Furthermore, proliferation starts in the media, progresses to the intimal shoulder region and endothelial coverage and shifts to the fibrous cap.

1166-14 Conditional Smooth Muscle Marker Expression by Smooth Muscle Cells From Transgenic Mice Expressing a Temperature-sensitive SV40 T Antigen Targeted to Smooth Muscle

L. Fan, K.L. March. *Krannert Institute of Cardiology and Roudebush VAMC, Indianapolis, IN, USA*

Control of smooth muscle differentiation and proliferation is of importance in the development and pathology of the vasculature. We have derived conditionally-transformed vascular smooth muscle cells (TsT-SMC) from transgenic mice expressing a temperature-sensitive mutant SV40 T antigen (tsA58) under control of the mouse vascular SMC α-actin promoter. These cells maintain α-actin expression despite extensive passaging. We hypothesized that such cells might also maintain expression of other smooth muscle cell markers; and that cell cycle exit at 39.5°C would be accompanied by increased expression of differentiation markers as well as negative cell cycle regulators. Expression of these genes was evaluated in cell populations and clones (n = 5) under permissive (33°C) and restrictive conditions, using immunoprecipitation, immunoblotting, and RNase protection assays. Expression of the smooth muscle markers, myosin heavy chain (MHC) 204, h-caldesmon, and smooth muscle calponin were identified in these cell types at 33°C; and MHC-204 was found to increase 3-fold at the level of RNA as well as protein, upon shift to 39.5°C. Immunoprecipitation/immunoblotting demonstrated the presence and association with T-antigen of each of the cell cycle regulator genes pRB, p107, p130/RB2, and p53 at 33°C. Shift to 39.5°C was accompanied by a 3-fold increase in level of hypo-phosphorylated pRB, without a measurable change in p107 or p130 expression or phosphorylation. p53 protein, however, was markedly reduced. These data demonstrate that TsT-SMC possess the capability for inducible differentiation in conjunction with cell cycle exit, and suggest that pRB may play a role in both proliferation and differentiation control in this cell type. TsT-SMC may provide an *in vitro* model for study of growth and differentiation in vascular smooth muscle.

1166-15 Coronary Artery Apoptosis Is Increased in a Porcine Model of Experimental Hypercholesterolemia

G. Sangiorgi, D. Hasdai, L.G. Spagnoli[1], H.M. Kwon, C. McKenna, D.R. Holmes, Jr., R.S. Schwartz, A. Lerman. *Mayo Clinic and Mayo Foundation, Rochester, MN, USA; [1]Institute of Pathology, University of Rome Tor Vergata, Rome, Italy*

Background: Apoptosis is a mode of cell death in which single cells are deleted in the midst of living tissue. Increased expression of apoptotic cells has been recently demonstrated in *in vitro* models of hypercholesterolemia (HC). However, the effect of HC on apoptosis rate *in vivo* is still unknown.

Methods: Pigs were sacrificed after consuming either a high-cholesterol diet for 10–16 weeks (n = 17) or normal diet (n = 9). Coronary sections were stained with hematoxylineosin and elastic van Gieson and the intimal, media and adventitia area were measured by morphometric analysis. Apoptosis was evaluated by TUNEL staining, confocal microscopy with propidium iodide, and transmission electron microscopy.

Results: Plasma cholesterol level were significantly higher in the cholesterol-fed animals compared to controls (342 ± 20 mg/dL vs. 89 ± 9 mg/dL, p < 0.001, respectively). Although the intima/media ratio was greater in hypercholesterolemic pigs (0.049 ± 0.010 vs. 0.21 ± 0.150, p = 0.07), atheromatous plaque were not evident. TUNEL staining was positive in 11 of 17 hypercholesterolemic animals (65%), primary in the intima (175 ± 58 cells/mm^2) and adventitia (107 ± 51 cells/mm^2), with few cells detected in the media (5 ± 1 cells/mm^2). No apoptotic cells were present in the animal fed with normal diet. Different morphological stages of apoptosis were evident by confocal microscopy and transmission electron microscopy.

Conclusion: This study demonstrates that experimental HC is characterized by coronary apoptosis prior to lesion formation, primary localized within the intima and adventitia layers of the vessel wall. This study suggests that apoptotic process may be important in the structural changes associated with early coronary atherosclerosis.

1166-16 Characterization of a Negative Cis-Element in the Rat Smooth Muscle α-Actin Promoter

F. Jung, B. Wei, D. Johnson, G.K. Owens, C.A. McNamara. *University of Virginia Health Science Center, Charlottesville, Virginia, USA*

Background: Identification of the regulators of smooth muscle cell (SMC) gene expression is critical for the understanding of SMC phenotype in various vascular diseases. Previous studies revealed negative transcriptional activity within a region of the rat smooth muscle (SM) α-actin promoter (−125 to −271). Here we characterize a highly conserved sequence (TGTTTATCCCCA) within this region.

Methods: DNAse I footprinting analysis and electrophoretic mobility shift assays using a 21 bp promoter fragment containing the conserved sequence as well as specific mutations (TacTTATCtagA, TGTTT ggg-CCCA, TGTTTggg-CCCA, TGTTTATCtagA and TacTTATCtgA) were performed. Transient transfection experiments in rat aortic smooth muscle cells using a 271 bp promoter fragment (wild type and the specific mutation listed above) in a luciferase reporter plasmid were done to assess functional properties of this conserved sequence.

Results: DNAse I footprinting analysis of this promoter region demonstrated protein binding to the conserved sequence TGTTTATCCCCA by one or more factors from rat aortic nuclear extracts. Gel shift experiments confirmed that binding of those nuclear factors to the 21 bp promoter fragment containing the conserved sequence was specific. Specific mutations abolished nuclear factor binding. Transient transfections of rat SM α-actin promoter/reporter gene constructs demonstrated that each specific mutation was sufficient to increase transcriptional activity, when transfected into rat SMC's.

Conclusion: Our studies demonstrate that nuclear proteins bind a 10bp negative cis-element within the rat SM α-actin promoter. Further characterization and isolation of the nuclear binding factors may provide important insights into the molecular mechanisms that regulate SM α-actin expression in SMC's in vascular diseases.

1166-17 Nitric Oxide Inhibits Proliferation of Human Aortic Vascular Smooth Muscle Cells: Role of Cell Cycle Regulatory Proteins

F.C. Tanner, T.F. Lüscher. *Cardiology, University Hospital Zürich; Physiology Institute, University Zürich-Irchel, Switzerland*

We studied the effect of nitric oxide (NO) on proliferation of human aortic vascular smooth muscle cells (VSMC). The nitric oxide donor DETANO (10^{-5} M–10^{-3} M) inhibited proliferation to both 10% serum and 10 ng/ml PDGF BB in a concentration-dependent manner with 100% inhibition at the highest concentration. Unlike fresh DETANO, the disintegrated NO donor did not inhibit proliferation. Trypane blue uptake was not increased upon treatment with DETANO. However, propidium iodide staining revealed that there was a slightly higher number of hypodiploid cells after exposure to the NO donor (7.8% vs. 1.6% under control conditions). Analysis of the cell cycle distribution demonstrated that the cells exposed to DETANO arrested in the G1 phase (82% vs. 64% under control conditions). In contrast to the NO donor, 8-bromo-cGMP reduced proliferation rates by only 24% even at 10^{-3} M, while both forskolin and 8-bromo-cAMP completely inhibited proliferation under these conditions. The cyclin-dependent kinase inhibitors (cki) p21 and p27 were induced after treatment with DETANO, while the cyclin-dependent kinase 2 (cdk2) level declined. In contrast, expression of cyclin E did not change. Consistent with these findings, activity of cdk 2 was blunted after treatment with the NO donor. Our data demonstrate that NO is an inhibitor of VSMC proliferation. This inhibition is primarily related to G1 arrest, while a small reduction in cell number due to a weak toxic effect can not be excluded. cGMP is only in part mediating the action of NO on proliferation. The G1 arrest is due to induction of the cki p21 and p27 and a concomitant decrease in cdk2 leading to inhibition of cdk activity.