Reverse Regulation of Endothelial Cells and Myointimal Hyperplasia on Cell Proliferation by a Heatshock Protein-Coinducer After Hypoxia

Laszlo Denes, PhD; Zoltan Bori, MS; Eva Csonka, DSc; Laszlo Entz, MD, PhD; Zoltan Nagy, MD, DSc

**Background and Purpose**—Myointimal hyperplasia (MIH) cells are related to permanent upregulated proliferation as tumor-like cells. The aim of this study is to assess whether treatment of cells after hypoxia by Iroxanadine heat-shock protein (HSP-coinducer) predicts recovery through cell proliferation.

**Methods**—Vascular smooth muscle cells (VSMC) and brain capillary endothelial cells (HBEC) were isolated from human origin and MIH-cells from early carotid restenosis after surgery. Cell proliferation was quantified by bromuridine (BrdU) incorporation after hypoxia/reoxygenation. HSP72 and cyclin-dependent kinase (CDKN1A) mRNA expression was assessed by reverse transcription-polymerase chain reaction (PCR) and cell cycle distribution by flow cytometry (FACS) analysis.

**Results**—After hypoxia/reoxygenation, the proliferation of MIH-cells increased, whereas endothelial cells decreased (MIH: 0.266±0.016 versus 0.336±0.024; P<0.05; HBEC: 1.249±0.10 versus 0.878±0.11; P<0.05). Whereas augmented proliferation of MIH-cells was reduced (40% to 45%) by HSP-coinducer, diminished HBEC proliferation increased (46.2%). Stress-activated-protein-kinase (SAPK)p38-dependent cell cycle redistribution was generated by an increase in HSP72 and CDKN1A mRNA levels in MIH-cells.

**Conclusions**—The 2 key players of early restenosis (MIH, EC) were oppositely regulated and correspondingly after treatment by HSP-coinducer reverse recovered. Drug candidate may have therapeutic potential in (re)restenosis. *(Stroke. 2008;39:1022-1024.)*

**Key Words:** carotid stenosis ■ endothelium ■ heatshock-protein ■ proliferation ■ smooth muscle cells

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Early restenosis (6 to 12 months after intervention) resulting from myointimal hyperplasia (MIH) is related to upstream proliferation of SMCs and down-regulated endothelial function that may be related to clinical symptoms. Therefore, strategies have been proposed to prevent early restenosis by agents and newly carotid artery stenting. Recently, drug-eluting stents is an alternative therapy still with problems.

Iroxanadine (Cytrx; 5, 6-dihydro-5- (1-piperidinyl) methyl-3-(3-pyridil)-4H-1, 2, 4-oxadiazine), originally synthesized by Biorex-Hungary (BRX-235), is a drug candidate with a marked cytprotection by heatshock protein (HSP)-coinduction.¹ This molecule has been proposed to stimulate the migration of ECs via p38 stress-activated-protein-kinase (SAPK) and enhanced expression of HSPs and heat-shock transcription factor 1.¹ However, no data were published according to this HSP-coinducer focusing on the effect regarding MIH.

We will show how BRX-235 interferes with MIH, human brain endothelial cells (HBEC) and vascular smooth muscle cells (VSMC) under hypoxia/reoxygenation.

**Methods**

The following cells were isolated from human origin: VSMCs/carotid wall;² brain capillary endothelial cells (HBEC)/cut of brain gray matter³ from accidental cadaver donors, and MIH-cells/segment of operated artery.

Cultivated cells were exposed to hypoxia (for 1 hour) in Billups-Rothenberg chamber and reoxygenated for overnight. Before hypoxia the endothelial cells were starved overnight by serum deprivation (1%).

MIH cells were isolated and cultured from surgical specimens of early restenosis. The 7 patients (age 62 to 78) were operated (6 to 12 months) after the first endarterectomy, whereas the second interventions were patch plastic, or interposition. No patients were lost and no neurological events occurred because of surgery.

Permission to isolate cells of human origin was granted by the Ethical Committee of the Hungarian Ministry of Health (595/98/KO and 73/1998/ETT). Materials were obtained primarily from Sigma.

Cell cultures were treated with HSP-coinducer (10⁻⁸-10⁻⁶ mol/L) next exposed to bromuridine (bromuridine [BrdU]/Roche Diagnostics Kit) overnight, the amount of incorporated BrdU measured by ELISA-reader. Cell cycles of MIH-cells were measured by flow cytometry (fluorescence activated cell sorter [FACS] Calibur).

Total cellular RNA was prepared and reverse transcription performed, the cDNA synthesized, 28S rRNA (forward and reverse
Acc. No.: U13369), amplified cyclin dependent kinase (CDKN1A)/WAF-1 (forward and reverse Acc. No.: L25610) and HSP72 cDNA (forward and reverse Acc. No.: L12723) specific primer pairs were designed. The amplified reverse transcription-polymerase chain reaction (PCR) products were visualized and demonstrated on figure.

Statistical analyses were performed by 1-way ANOVA and significance calculated by Student t test.

**Results**

The proliferation rate of nonpathological cells: VSMC (n=4) and HBECs (n=6) decreased (21.2%, P<0.05, and 30.7%, P<0.009) after hypoxia/reoxygenation. Conversely, the activity of cultured MIH (n=7) cells increased (31.4%, P<0.001; Figure 1a). Using HSP-coinducer at normoxia, the upregulated proliferation rate of MIH-cells reduced (22.8 ± 1.1 P=0.048) similarly after hypoxia/reoxygenation (0.1 μmol/L to 1 μmol/L Iroxandine 37.1±2.5% P=0.007, 34.3±3.0% P=0.009; Figure 1b). Contrarily, the downstreamed proliferation rate of HBECs was upregulated while the test drug did not elicit any effect on VSMC (Figure 1c).

In MIH cell population the subpopulation of G0/G1 phase cells represented the highest percentage, but no change was observed after HSP-coinduction. The number of S phases of cells downstreamed (17.8±0.7/12.3±1.0%; P=0.008) and G2/M increased (10.2±1.1/17.0±0.9%; P=0.0078; Figure 2a, b). An opposite effect appeared by SAPK p38 inhibitor (SB203580 1 μmol/L). The number of S phase cells increased (17.0±0.9 /21.2±1.0%; P=0.046) except for G2/M subpopulation (8.2±0.8 /9.8±1.0%; Figure 2a). The HSP-coinducer (1 μmol/L) counteracted the effect of SB203580 (1 μmol/L) and increased the cell ratio of G2/M phase and decreased the upregulated cell ratio of S phase (Figure 2b). Thus, the HSP-coinducer induced p38-dependent cell cycle redistribution on cell subpopulations.

In VSMC after hypoxia/reoxygenation, HSP72 and CDKN1A mRNA levels did not change (data not depicted). In HBEC treated with HSP-coinducer (for 5 days) after hypoxia/reoxygenation both the HSP72 mRNA (4.9-fold) and the CDKN1A mRNA (4.7-fold) decreased. In MIH a slight upregulation of HSP72 (2.6- to 3.0-fold) and CDKN1A mRNA level (1.5- to 1.9-fold) was measured. The HSP-coinducer treatment reversed the effect of hypoxia/reoxygenation (Figure 3).

**Figure 1.** Proliferation of HBEC, VSMC and MIH-cells by incorporated BrdU. Proliferation (after 1 h hypoxia, 24 h reoxygenation) was measured by ELISA, calculated (mean ± SEM). Difference of significance are *P<0.05, **P<0.01 and ***P<0.005 by Student t test. a, Proliferation rates of 3 cells are compared on normoxia (dark bars); on hypoxia (open bars) and Hypoxia; SD=Serum Deprivation; b, treated MIH cell proliferation on normoxia and on hypoxia/reoxygenation. Control cells (dark bars), treated by HSP-coinducer: 0.1 μmol/L (open lines), 1.0 μmol/L (ruled bars) and 10 μmol/L (dotted bars); c, proliferation of HBEC and control VSMC after hypoxia/reoxygenation are depicted at normoxia (dark bars) and after hypoxia/reoxygenation (open bars). The HSP-coinducer treatment is demonstrated as shown above.
Discussion

A definitive therapy on restenosis and in-stent stenosis, especially targeting VSMC as well as endothelial cell dysfunction, is warranted.4 Cultured MIH-cells with hypoxia/reoxygenation are used as model of restenosis to test drug actions. In between MIH-cells and endothelial cells reverse regulation was observed on cell proliferation particularly after hypoxia. The proliferation of nonpathological cells (VSMC and EC) decreased while pathological MIH-cells greatly increased. This novel observation explains different mechanisms in early restenosis.

Drug-induced HSPs may have therapeutic effects under different pathological conditions.1 After treatment by HSP-coinducer, the augmented proliferation of MIH-cells decreased but the proliferation of deregulated EC cells was enhanced. Simultaneously, the expression of HSP72 and CDKN1A are upregulated in agreement with clinical reports where compromised endothelial cell proliferation and upregulated HSP72 expression was described in VSMC after transluminal balloon angioplasty.5

MIH-cells are characterized by augmented proliferation, similar to tumor cells. Induced by heat or stress proteins, tumor suppressor p53 inhibited cell growth by enhanced expression of CDKN1A/p21.6 While not in HBEC and VSMCs, CDKN1A/p21 upregulation was documented in the MIH cell culture after HSP-induction.

As we documented, MIH and endothelial cells, the 2 key players of early restenosis, were oppositely regulated. Iroxanadine, a HSP-coinducer, may prevent restenosis by its regulatory action.

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Disclosures

None.

References


Figure 2. Cell cycle of MIH-cells with HSP-coinducer SB203580 tested by FACS. a, Percentage of G2/M and S cells after HSP-coinducer or SB203580 treatment. b, G2/M and S cells after HSP-coinducer and SB203580.

Figure 3. HSP72 and CDKN1A mRNA expression on MIH and HBEC after HSP-coinduction. PCR were performed in 3 parallel tubes of the same cDNA samples. Lanes: 2 µL of cDNA from cells without HSP-coinducer 1 to 3 (EC) and 7 to 9 lines (MIH); Treated cells: 4 to 6 (EC) and 10 to 12 (MIH); 13 line: 2 µL negative control of cDNA (NC).
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