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INDUCTION OF HOUSEKEEPING GENE EXPRESSION AFTER SUBARACHNOID HEMORRHAGE IN DOGS

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Abstract

Changes in gene expression are commonly assessed relative to the expression of housekeeping genes, which are assumed to remain unchanged. We tested this assumption in cerebral arteries obtained from dogs 4 and 7 days after SAH had been created using the double hemorrhage model. Basilar arteries were removed and examined for expression of messenger ribonucleic acid (mRNA) levels using quantitative real-time polymerase chain reaction. Cross sections of basilar arteries were stained immunohistochemically for proliferating cell nuclear antigen (PCNA) and 4',6-diamidino-2-phenylindole (DAPI). Positively-stained cells were counted and numbers obtained were normalized to the cross-sectional area. Results were compared to normal dog basilar arteries contracted pharmacologically *in vitro*. SAH resulted in significant vasospasm ($P < 0.001$ for each, paired t-tests). There were significant increases in mRNA for β -actin (441%, $P = 0.01$), glyceraldehyde-3-phosphate dehydrogenase (566%, $P = 0.007$) and 18S ribosomal RNA (320%, $P = 0.025$) 7 days after SAH. Total mRNA was increased 7 days after SAH relative to genomic DNA (157%, $P = 0.009$). There were significant increases in the number of cells in the tunica media and adventitia of arteries after SAH and a significant decrease in the media after contraction *in vitro*. Cells in the tunica media and adventitia labeled with PCNA were significantly increased at both times after SAH. Transcripts for housekeeping genes are increased after SAH, making standardization to them potentially invalid. The increase is due to proliferation of cells in the adventitia and increased total mRNA in the media and adventitia.

Keywords

Cerebral vasospasm; gene expression; housekeeping genes; subarachnoid hemorrhage

INTRODUCTION

Cerebral vasospasm is a transient, delayed constriction of cerebral arteries that occurs after subarachnoid hemorrhage (SAH). Vasospasm has been difficult to prevent or reverse with vasodilators, however, leading to the suggestion that structural changes occur in the cerebral arteries and that these contribute to the narrowing (Bevan et al., 1987; Ohkuma et al., 2001;

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Borel et al., 2003). As early as 1980, Kassell and others hypothesized that vasospasm was due to proliferation of cells in the arterial wall (Peerless et al., 1980; Kassell et al., 1980) and this was followed by work both supporting (Smith et al., 1985; Kapp et al., 1985) and refuting this possibility (Pluta et al., 1992). During experiments assessing changes in gene expression in vasospastic arteries, we noted that levels of messenger ribonucleic acid (mRNA) for genes that are commonly used to standardize measurements to, such as β -actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18S ribosomal RNA (rRNA), was not constant. Transcript levels for these “housekeeping” genes may be altered in blood vessels in other diseases (Kumar and Owens, 2003). Furthermore, the arterial response to atherosclerosis, balloon angioplasty, systemic and pulmonary hypertension and possibly vasospasm after SAH may be accompanied by alteration of smooth muscle from a contractile to a proliferative, synthetic phenotype. Proliferative smooth muscle cells demonstrate increased rough endoplasmic reticulum (Halayko et al., 1997). While quantitative measurements of ribosomes have seldom been made, these changes suggest that even rRNA might be altered under some conditions. We therefore tested the hypothesis that vasospasm is associated with changes in mRNA for certain housekeeping genes after SAH. We counted cells in each layer of the arterial wall at various times after SAH to determine if changes were due to an increase in number of cells and to establish whether genomic deoxyribonucleic acid (gDNA) could be used as a housekeeping standard. The results call into question literature reporting changes in gene expression after SAH (Hino et al., 1996b; Sayama et al., 1998; Ohkuma et al., 1999; Onda et al., 1999; Suzuki et al., 1999; Ono et al., 2000; Miyagi et al., 2000; Aihara et al., 2001) and suggest that there is significant cell proliferation within certain layers of the arterial wall after SAH in dogs.

MATERIALS AND METHODS

Animal Model

17 mongrel dogs weighing 15–25 kg underwent baseline cerebral angiography and then were randomly assigned to serve as controls (n=7) or to undergo creation of SAH by injection of 0.5 ml/kg autologous, nonheparinized, arterial blood into the cisterna magna on the day of angiography (day 0) and then again 2 days later (day 2). SAH dogs underwent repeat angiography on day 4 (n=4) or 7 (n=7) after which they were euthanized. Methods for angiography, creation of SAH, monitoring of physiological parameters (blood pressure, heart rate, body temperature, arterial carbon dioxide and oxygen concentrations), standardization of angiography and preservation of tissue quality for histological and molecular analysis have been described (Macdonald et al., 1991; Macdonald et al., 1995; Aihara et al., 2004). All procedures on animals were performed under general anesthesia with endotracheal intubation and ventilation using sterile techniques and were approved by the Institutional Animal Care and Use Committee.

RNA Processing

Animals were euthanized under general anesthesia by exsanguination and transcardiac perfusion with 8 L ice-cold phosphate-buffered saline (PBS, pH 7.4) at physiological intraluminal pressure. This volume of perfusate completely cleared intraluminal blood and rapidly cooled the brain. After perfusion the brain was excised and placed in 4°C PBS. The arachnoid membrane surrounding the basilar artery was removed under an operating microscope. The endothelial layer was removed by passing an angiography guidewire of the same diameter as the artery through the arterial lumen and then flushing the artery with PBS. Basilar arteries were then separated from the brain and frozen in liquid N₂. RNA was extracted from individual basilar arteries with TRIzol (Gibco BRL, Life Technologies, Rockville, MD). RNase-free DNase (Promega, Madison, WI) was used to eliminate contamination with genomic DNA. RNA reverse transcription (RT) was catalyzed using a SUPER SCRIPT first-

strand synthesis system (Invitrogen Life Technologies, Carlsbad, CA). RNA (5 µg) was used for RT in a final volume of 10 µL. The reaction mixture consisted of 50 ng of random hexamers per µg of total RNA. Sensitivity of polymerase chain reaction (PCR) from first-strand cDNA was increased by using RNase H to remove the RNA template from cDNA following first-strand synthesis.

Genomic DNA Precipitation and Quantitative RT-PCR

After RNA extraction, DNA was precipitated from the organic phase of the TRIzol solution. Genomic DNA concentration was quantified in a Gene Spec III spectrophotometer (Hitachi Genetic Systems, Alameda, CA). Dog sequences for β-actin and GAPDH were obtained from the NCBI database (<http://www.ncbi.nlm.nih.gov/Entrez>). Primers, probes and lengths of PCR products were chosen to satisfy the requirements specified in Primer Express (version 1.5) software (Applied Biosystems, Foster City, CA) for quantitative RT-PCR (Table 1). Constitutively-expressed 18S ribosomal RNA (18S rRNA; TaqMan ribosomal RNA control reagent, Applied Biosystems) also was used as a possible housekeeping gene. Quantitative, real-time RT-PCR was then performed as previously described using a Sequence Detection System model 7700 (Applied Biosystems)(Aihara et al., 2004).

Immunohistochemistry, Contraction In Vitro and Cell Counting

For contraction of control, normal basilar artery *in vitro*, cross sections of medulla 5 mm thick with the basilar artery attached were placed in tissue baths containing Krebs-Henseleit buffer (in mmol/L: NaCl 123, KCl 5.9, CaCl₂ 2.5, MgCl₂ 1.2, NaHCO₃ 25, H₂SO₄ 1.2, NaH₂PO₄ 1.2 and glucose 11), bubbled with 95% O₂ and 5% CO₂ at 4°C. Prostaglandin F_{2α} (PGF_{2α}, 100 µm/L), was added to the bath. This concentration of PGF_{2α} was previously shown to produce maximal contraction of normal dog basilar artery under isometric tension (Macdonald, Zhang et al., 1995). Once contraction was confirmed by observation under a dissecting microscope, samples were fixed in 4% paraformaldehyde in PBS. Five mm segments of brainstem and basilar artery from SAH animals were fixed in identical fashion in order to exclude the possibility that cell counts in arterial cross sections could be systematically biased by differential longitudinal shortening of the arteries.

Cross sections (5 µm thick) of basilar arteries contracted *in vitro* and from animals 4 and 7 days after SAH were dehydrated, embedded in paraffin, and stained with primary antibodies to proliferating cell nuclear antigen (PCNA, Santa Cruz Biotechnology, Santa Cruz, CA) and CD31 (Santa Cruz). For counting cells, sections were mounted with VECTASHIELD Hard Set Mounting Medium (Vector Laboratories, Inc., Burlingame, CA) containing 4',6-diamidino-2-phenylindole (DAPI) to counterstain DNA. Labeled cells were counted in the entire artery on 5 cross sections of each artery for each marker by 2 independent and blinded observers and a mean cell count obtained for each artery. Cells were classified as being in the tunica adventitia, media or intima. Oblique sectioning through the artery could artifactually alter cell counts (Macdonald et al., 1991). This was prevented by careful orientation of the sectioning plane perpendicular to the long axis of the artery. Preliminary experiments showed that altering the sectioning plane by 5° did not significantly alter cell counts. Because the entire cross section was analyzed, stereological corrections to remove biases in counting were not used (Mayhew and Gundersen, 1996). The only artifact introduced would be that due to longitudinal shortening of the artery which is an accepted inherent limitation in similar studies (Mulvany, 1999). Arterial cross sectional areas including areas of adventitia and media were calculated by importing digital images of the cross sections into Adobe Photoshop (version 7.0, San Jose, CA) and analyzing using an image processing tool kit (version 4.0, Reindeer Graphics, Asheville, NC).

Statistical Analysis

Comparisons between multiple groups were made using analysis of variance (ANOVA) followed by pairwise t-tests if significant variance was found. Statistical analysis was performed using SigmaStat (SPSS, Chicago, IL) and Microsoft Excel (Redmond, WA). All results are expressed as means $\pm$ standard error of the mean.

RESULTS

Angiographic Vasospasm

SAH was associated with significant reduction in basilar artery diameter on days 4 ($52 \pm 4\%$ decrease, $P < 0.001$, paired t-test, $n = 4$) and 7 ($54 \pm 3\%$ decrease, $P < 0.001$, paired t-test, $n = 7$). There were no differences between groups in basilar artery diameter at baseline (ANOVA). There were no significant differences in physiological parameters (blood pressure, heart rate, body temperature, arterial PO_2 , arterial and end-tidal PCO_2) between groups at each time or within groups over time (data not shown).

RNA Levels

Target gene expression levels were expressed as absolute amounts normalized to the total genomic DNA (gDNA) in the same samples. There were significant increases in RNA for all candidate housekeeping genes 7 days after SAH including β -actin (441%, $n = 7$, $P = 0.01$, all unpaired t-tests, Fig. 1), GAPDH (566%, $n = 7$, $P = 0.007$) and 18S rRNA (320%, $n = 7$, $P = 0.009$, Fig. 1). In addition, there was a significant increase in total RNA measured as the amount of cDNA relative to genomic DNA (157%, $n = 7$, $P = 0.009$).

Cell Counts and Cross-Section Areas

Labeling of nuclear DNA with DAPI allowed accurate counting of the total number of cells in the tunica media and adventitia of each arterial cross section. Cell counts in the tunica media showed that there were no significant changes 4 (629 ± 134 , $n = 4$) and 7 days (691 ± 91 , $n = 7$) after SAH compared to control basilar artery (507 ± 38 , $n = 7$, ANOVA, Fig. 2). Area of the tunica media also was not significantly different (day 0: 221875 ± 56406 , day 4: 171108 ± 50322 , day 7: $172134 \pm 40757 \mu m^2$, $n = 4-7$, ANOVA). When cell counts were expressed as cells/ μm^2 , there were significant increases 4 (0.00366 ± 0.00012 cells/ μm^2) and 7 (0.00342 ± 0.00013) days after SAH compared to control (0.00252 ± 0.00013 , $n = 4-7$, $P < 0.001$, ANOVA).

Cell counts in the tunica adventitia showed a significant increase 4 days after SAH with a significant pairwise difference between days 0 and 4 (day 0: 519 ± 57 , day 4: 882 ± 141 , day 7: 652 ± 66 , $p = 0.004$, ANOVA, Fig. 3). There were no significant changes in adventitia areas (day 0: 59654 ± 7073 , day 4: 54201 ± 4665 , day 7: $50714 \pm 3611 \mu m^2$). Cell density in the adventitia was significantly higher 4 (0.0163 ± 0.002 cells/ μm^2) and 7 (0.0129 ± 0.0009) days after SAH compared to control (0.00875 ± 0.00043) with no significant pairwise difference between days 4 and 7 ($p = 0.004$, ANOVA, Fig. 3).

For arteries contracted *in vitro*, there were no differences between control basilar artery and artery contracted *in vitro* in tunica media cell counts (control: 516 ± 56 , contracted: 584 ± 63 cells, $n = 4$, Fig. 2) or areas (control: 133987 ± 42870 , contracted: $210096 \pm 50803 \mu m^2$, $n = 4$). There was a significant decrease in cell density after contraction *in vitro* (control: 0.00391 ± 0.00027 , contracted: 0.00283 ± 0.00007 cells/ μm^2 , $n = 4$, $P = 0.008$, unpaired t-test). There were no significant differences in adventitia cell counts, areas or cells/ μm^2 (control: 609 ± 85 , 58037 ± 14158 , 0.0116 ± 0.0026 and contracted: 535 ± 52 cells, $40925 \pm 9408 \mu m^2$, 0.0141 ± 0.0016 cells/ μm^2 , respectively, $n = 4$, Fig. 3).

The number of cells staining positive for PCNA was significantly increased in the tunica media and adventitia 7 days after SAH ($P < 0.05$, $n = 4-7$, ANOVA, Fig. 4) with significant pairwise differences for each layer between day 7 and control (Table 2). Qualitative assessment showed relatively more intense staining in cells in the adventitia than generally seen within cells in the media. Arteries contracted *in vitro* demonstrated no differences from control arteries (Table 2).

DISCUSSION

Quantitative real-time RT-PCR demonstrates that levels of mRNA for housekeeping genes, such as β -actin, GAPDH and 18S rRNA, are significantly increased in vasospastic arteries 7 days after SAH in dogs when standardized to the amount of gDNA present. The levels of mRNA for these genes has been assumed to be stable in some prior studies (Hino et al., 1996a; Sayama et al., 1998; Ohkuma et al., 1999; Onda et al., 1999; Suzuki et al., 1999; Ono et al., 2000; Miyagi et al., 2000; Aihara et al., 2001). If housekeeping gene mRNA increases, then genes reported to have increased expression might actually have been increased even more. For genes whose mRNA was reported to be unaltered or decreased, it would be impossible to say whether they were actually altered or not. Changes in housekeeping genes are reported in other diseases as well and the present results suggest that relevant standards should be determined specifically in each disease and model (Bond et al., 2002).

Previous studies have examined changes in housekeeping genes after SAH. Hino and colleagues found no difference in β -actin mRNA between vasospastic and normal monkey middle cerebral arteries although the comparison to normal arteries in the same animal does not rule out the possibility that a systemic change in β -actin occurred (Hino et al., 1996b). Onda, et al., reported that analysis of dots on a microarray for β -actin, GAPDH and 4 other housekeeping genes (ubiquitin, α -tubulin, 23 kDa highly basic protein and ribosomal protein S9) were of equal intensity in normal dog basilar artery and dog basilar artery 7 days after SAH (Onda et al., 1999). The array analysis used was not as quantitative as the methods used here. There was no change in GAPDH 7 days after SAH in dogs (Ohkuma et al., 1999). In the same model, Ohkuma and colleagues examined β -actin and changes in its 3' untranslated region that are considered to be nonspecific markers of vascular remodeling (Ohkuma et al., 2001). There was a significant increase in mRNA for β -actin normalized to 18S or 28S rRNA. We also found, however, an increase in 18S rRNA. Smooth muscle cells develop increased rough endoplasmic reticulum when they alter phenotype in response to arterial injury although it is not clear if the number of ribosomes increases (Halayko et al., 1997).

Genomic DNA was used as a standard. This also may have limitations because DNA content of smooth muscle may vary and an increase in mRNA in the entire sample examined would not be detected if it were due to an increase in the number of cells present. These results are valid, however, even if genomic DNA increased in some cells since this could only serve to reduce the measured increases in housekeeping gene mRNA. Regarding DNA content of smooth muscle, nothing is known about changes occurring after SAH but systemic hypertension in experimental animals and humans is associated with smooth muscle hypertrophy in the media and development of polyploidy (Owens, 1995). The time course in hypertension models is slow with a 20–30% increase in cell protein content over 3 months in diploid smooth muscle cells. On the other hand, various contractile agonists can acutely increase protein synthesis and lead to cellular hypertrophy which are 2 features associated with hypertension and therefore, polyploidy (Owens, 1995).

Regarding cell number, the present results document increased cells in the media and adventitia of arteries with vasospasm after SAH. This is substantiated by increased numbers of cells demonstrating immunoreactivity to PCNA, a protein expressed when cells enter S phase and

may be proliferating (Egashira et al., 2002). These findings are consistent with those of Borel, et al., who found an increase in PCNA-positive cells, mainly in adventitial fibroblasts of arteries surrounded by blood clot 3 to 72 hours after SAH in mice (Borel et al., 2003). Vasospasm was not documented in these experiments. The authors suggested that the proliferation of these cells was similar to that occurring in other diseases like atherosclerosis and pulmonary hypertension. Vasospasm was suggested to share with these diseases certain common features such as smooth muscle proliferation, wall thickening, synthesis of extracellular matrix and reduced arterial compliance (Peerless et al., 1980; Kassell et al., 1980). Other investigators reported proliferation of intimal cells during vasospasm (Kapp et al., 1985) and synthesis of type 5 collagen that is associated with smooth muscle proliferation (Smith et al., 1985) whereas Pluta and colleagues did not find substantial cell proliferation during vasospasm in a primate model (Pluta et al., 1992). There are, however, some differences between vasospasm and these diseases. While reduced arterial compliance is well documented during vasospasm, presence of the other features has not been rigorously shown (Bevan et al., 1987; Macdonald et al., 1995).

Vasospastic arteries have reduced compliance and this has been suggested to indicate that there has been vascular remodeling (Bevan et al., 1987; Macdonald et al., 1995). Vascular remodeling is an active process by which blood vessels respond to alterations in hemodynamics and involves cell growth, death and migration and production and degradation of the extracellular matrix (Gibbons and Dzau, 1994). It frequently is accompanied by alteration in smooth muscle from a contractile to a synthetic phenotype. This phenotype change is accompanied by activation of DNA, RNA and protein synthesis and reduced expression of smooth muscle contractile proteins such as α -actin and increased expression of β -actin (Etienne et al., 1998). In addition to the evidence reviewed above, several other lines of evidence support this supposition including the decrease in α -actin that may occur in arteries after SAH (Gomis et al., 2000).

Previous measurements of arterial wall areas are consistent with the present results. There were no significant increases in areas of vasospastic arteries after SAH in monkeys (Macdonald et al., 1991). Ohkuma and colleagues found a significant increase in area of the tunica media 7 and 14 days after SAH in dogs but no change in the number of cells in this layer (Ohkuma et al., 2001). The reasons for these discrepancies may relate to methodological differences.

Additional studies are needed to determine the role of cell proliferation and arterial remodeling in vasospasm. Reduced compliance as well as intimal proliferation and fibrosis persist well beyond the clinically-important angiographic phase of vasospasm (Vorkapic et al., 1991; Macdonald et al., 1992). Therefore, it is not clear whether these changes are an epiphenomenon occurring secondary to the SAH or vasospasm or whether they contribute in some way to the arterial narrowing. We did not identify the type of cells labeled with PCNA. Changes in mRNA were not confirmed at the protein level. Proliferative smooth muscle may synthesize more protein but protein synthesis, measured by ribosome aggregation rate and amino acid incorporation *in vitro*, was significantly reduced in vasospastic arteries from dogs (Oka et al., 1996).

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Figure 1.

Bar graphs of absolute expression level of mRNA for β -actin (A), GAPDH (B), 18S rRNA (C) and the ratio of total RNA as measured by cDNA derived from reverse transcription to genomic DNA (D). There were significant increases in mRNA for each gene and in the total amount of RNA present (unpaired t-tests, $n=7$ per measurement). Absolute expression values are calculated as $C_T/[cDNA] \times (RNA_{Total}/gDNA_{Total})$.

Figure 2.

Bar graphs of total cell counts in tunica media, media area and cells per μm^2 media area of control dogs (day 0) compared to dogs 4 and 7 days post-SAH (**A, C, E**) and of control dogs compared to basilar arteries contracted *in vitro* (**B, D, F**). There was a significant increase in cell density on day 4 and 7 compared to control ($p < 0.001$, ANOVA, $n=5$ sections from $n=4-7$ dogs per group) and a significant decrease in cell density in arteries contracted *in vitro* ($P=0.008$, unpaired t-test, $n=4$ per measurement).

Figure 3.

Bar graphs of total cell counts in tunica adventitia, adventitia area and cells per μm^2 adventitia area of control dogs (day 0) compared to dogs 4 and 7 days post-SAH (**A, C, E**) and of control dogs compared to basilar arteries contracted *in vitro* (**B, D, F**). The significant changes were an increase in cells on day 4 after SAH and an increase in density of cells 4 and 7 days after SAH (P=0.004, ANOVA, n=5 sections from n=4–7 dogs per group).

Figure 4.

Photomicrographs of CD31 (top row), DAPI (middle row) and PCNA (bottom row) staining of cross sections of control dog basilar artery and basilar artery 4 and 7 days after SAH. CD31 staining is visible in the endothelium of a normal dog basilar artery (top left) whereas it is absent after deliberate denudation of the endothelium (top right). DAPI staining shows increased cells in the tunica media and adventitia 4 and 7 days after SAH. There are few cells labeled in the tunica media and more cells labeled in the tunica adventitia of arteries 4 and 7 days after SAH compared to control.

Table 1
Oligonucleotide sequences of dog-specific primers and probes used for real-time quantitative RT-PCR

Gene	Sense Primer	Probe	Antisense Primer	Size (Base Pairs)	Accession Number
β-actin GAPDH	CCGCCTATTCCAGGATTCTCT	TGAACCCAGTTGTCAATTCTGTCTTGCCG	GGACCTTCCCAACCCTGTTAG	88	AF021873
	CACCCCAATGTATCAGTTGTG	CCTGGAGAAAGCTGCCAAATATGACGACA	TGCCCTGCTTCACTACCTTCTTG	88	AB038240

Table 2

Cell counts and wall areas

% Cells with PCNA	Control (n=7)	Day 4 (n=4)	Day 7 (n=7)	In Vitro Uncontracted (n=4)	In Vitro Contracted (n=4)
Tunica media	0.03±0.03	8.0±8.0	14±6 [*]	0.02±0.01	0.03±0.02
Tunica adventitia	6.6±3.3	16.3±11.2	22.2±4.7 [*]	5.8±2.6	4.5±3.1

* P<0.05, ANOVA, day 7 significantly different from day 0 but no significant difference between days 4 and 7.