DNA damage and repair in a model of rat vascular injury

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ABSTRACT

Restenosis rates following vascular interventions still limit their long-term success. Oxidative stress plays a relevant role in this pathophysiological phenomenon, but less attention has been devoted to its effects on DNA damage and to the subsequent mechanisms of repair. In the present study, we analysed in a model of arteriotomy-induced stenosis in rat carotid arteries the time-dependent expression of DNA damage markers and of DNA repair genes, together with the assessment of proliferation and apoptosis indexes. The expression of the oxidative DNA damage marker 7,8-dihydro-8-oxo-2′-deoxyguanosine was increased at 3 and 7 days after arteriotomy, with immunostaining distributed in the injured vascular wall and perivascular tissue. Expression of the DNA damage marker phospho-H2A.X was less relevant, but increased from 4 h to 7 days after arteriotomy, with immunostaining prevalently present in the adventitia and, to a lesser extent, in medial smooth muscle cells at the injury site. RT (reverse transcription)–PCR indicated a decrease in eight out of 12 genes involved in the DNA repair machinery we selected from 4 h to 7 days after arteriotomy, with the exception of an increase in the MutYh and Slk genes (P < 0.05). Western blot analysis revealed a decrease in p53 and catalase at 3 days after arteriotomy (P < 0.05). A maximal 7 % of BrdU-positive cells in the endothelium and media occurred at 7 days after arteriotomy, whereas the apoptotic index peaked at 3 days after injury (P < 0.05). In conclusion, our results highlight a persistent DNA damage, presumably related to a temporary decrease in the expression of the DNA repair machinery and of the antioxidant enzyme catalase, playing a role in stenosis progression.

INTRODUCTION

The present strategies for severe atherosclerosis in arteries rely on angioplasty procedures, directional coronary atherectomy and bypass surgery. In reference to the occlusion of extra-cardiac arteries, the carotid artery can be subjected to endarterectomy or angioplasty to normalize impaired cerebral haemodynamics. Previous randomized trials have not revealed to date a substantial advantage of the more recently introduced carotid

Key words: apoptosis, carotid artery, DNA repair, oxidative stress, proliferation, restenosis, vascular injury.

Abbreviations: ATM, ataxia telangiectasia mutated; AngII, angiotensin II; BER, base excision repair; BrdU, bromodeoxyuridine; CAT, catalase; DSB, DNA double-strand break; ECM, extracellular matrix; EC, endothelial cell; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MER, mismatch excision repair; NER, nucleotide excision repair; 8-oxo-dG, 7,8-dihydro-8-oxo-2′-deoxyguanosine; ROS, reactive oxygen species; RT, reverse transcription; SMC, smooth muscle cell; SOD, superoxide dismutase; Mn-SOD, manganese SOD; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling.

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stenting with respect to endarterectomy, which remains the primary choice for treatment of severe symptomatic carotid stenosis [1].

Restenosis is considered as an excessive reparative reaction to vascular injury occurring during the above-mentioned revascularization procedures. The rate of this pathophysiological phenomenon is still high in Western countries [2]. The main factors in restenosis progression are cell proliferation and migration, neointima formation, cell transdifferentiation, inward remodelling and the related impaired ECM (extracellular matrix) metabolism. In addition, the intra-operative damage to adventitia and to vasa vasorum and nervous fibres it contains can have deleterious effects on restenosis [3], as vascular injury can stimulate the shift of adventitial fibroblasts to activated myofibroblasts [4], which in turn can exacerbate vessel remodelling and contribute to neointima formation after vascular injury. An in-depth knowledge of the different cell types and molecular pathways contributing to restenosis could lead to the establishment of novel protocols for the therapy of restenosis.

ROS (reactive oxygen species) include a number of oxygen-derived molecules that act as rapidly diffusing short-lived messenger molecules, with a recognized role in signal transduction. ROS can be generated by a number of different enzymes, including NADPH oxidases, whose activity is counterbalanced by other molecules, such as glutathione, SOD (superoxide dismutases), CAT (calatalase) and peroxidases, in order to preserve the cellular redox balance [5]. ROS can induce a potential damage of cellular elements. The level of ROS in the vascular wall can be increased by different stimuli, including shear stress [6], inflammation and growth signals. For example, AngII (angiotensin II) is a potent activator of NADPH oxidase in the cardiovascular system and is able to increase the production of ROS [7].

ROS have been implicated in hypertension and other vascular diseases, as the intracellular signalling cascades stimulated by ROS play an important role in their pathogenesis. Free radicals do not contribute to diseases solely as random damaging agents, but can serve as signalling molecules and modify transcription factors at different levels and, thus, have a direct role in transcriptional regulation [8]. ROS-induced stimulation of protein phosphorylation pathways modulates transcription factor activities and gene expression, which results in a variety of responses such as cell growth, differentiation or apoptosis [9,10]. The particular response observed depends on the cell type and the concentration and duration of ROS production. ROS production has been detected in vascular SMCs (smooth muscle cells) and ECs (endothelial cells) at the injury site, but it may also derive from infiltrating leukocytes.

Although the general cytotoxic effects of ROS in cardiovascular diseases has been well assessed [11], with particular relevance to atherosclerosis and myocardial infarction, only a few studies have specifically addressed the molecular events related to DNA damage and repair in restenosis [11a].

In the present study, we aimed to analyse in a well-characterized model of rat carotid arteriotomy the time course of two robust markers of DNA damage and repair, 8-oxo-dG (7,8-dihydro-8-oxo-2′-deoxyguanosine) and the phosphorylation of the H2A.X histone isoform (phospho-H2A.X), together with the determination of apoptosis and proliferation indexes, the Western blot analysis of Mn-SOD (manganese SOD), CAT and p53, proteins known to be involved in ROS metabolism, DNA repair and apoptosis, and the RT (reverse transcription)–PCR expression profile of a panel of 12 DNA repair-related genes.

Overall, the findings of the present study highlight a marked and persistent oxidative DNA damage in parallel with cell proliferation and apoptosis, and relevant differences in the expression profiles of genes involved in the DNA repair machinery, possibly reflecting distinct roles for these factors in the molecular events following arterial surgical injury.

**MATERIALS AND METHODS**

**Animals**

Studies were carried out on 12-week-old male Wistar rats (230–250 g; Charles Rivers). All animals were handled in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH publication No. 85-23, revised 1996). All protocols were approved by the Animal Care and Use Committee of the Second University of Naples. Rats were acclimatized and quarantined for at least 1 week before undergoing surgery.

**Vascular Injury**

Arteriotomy of rat common carotid artery was performed as described previously [12]. Briefly, a plastic Scanlom clamp for coronary artery grafting was placed for 10 s on the carotid artery causing a crushing lesion to the vessel. At the same point where the clamp was applied, a 0.5 mm longitudinal incision was done on the full thickness of the carotid artery. The incision did not cross to the other side of the vessel. Haemostasis was obtained with a single adventitial 8.0-gauge polypropylene stitch. Once bleeding had stopped, the carotid artery was carefully examined and blood pulsation was checked distally to the incision.

**RNA extraction and RT–PCR analysis**

Total RNA was extracted from injured rat carotid arteries at 4 h and at 3 and 7 days after arteriotomy (n = 4 rats, for each time point) and from uninjured rat carotid arteries.
(n = 4) using the RNAeasy minikit (Qiagen), according to the manufacturer’s instructions. RNA was treated with DNase (Qiagen) to remove DNA contamination. RNA concentration was measured using an ND-1000 spectrophotometer (NanoDrop Technologies). RNA integrity was verified by electrophoresis on a denaturing 1 % (w/v) agarose gel. The absence of residual DNA was verified by PCR on total RNA without RT.

cDNA was generated from 200 ng of each RNA sample. RT was done at 42 °C for 1 h in the presence of random hexamers and MMLV (Moloney-murine-leukaemia virus) reverse transcriptase (Finnzymes). GenBank® sequences for rat mRNAs and Primer Express software (Applied Biosystem) were used to design primer pairs for the genes related to DNA repair listed in Table 1. Expression data were normalized with respect to the level of the housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase).

Primer pairs were chosen to yield 100–150 bp PCR products and were validated by running the PCR products on an agarose gel to confirm a single band. Melting curves from 65 to 94 °C were also generated to determine whether there were any spurious amplification products. Each RT–PCR reaction was repeated at least three times. The real-time PCR assays were run on an Opticon 4 machine (Bio-Rad Laboratories). Reactions were performed in quadruplicate, according to the manufacturer’s instructions, using the SYBR Green PCR master mix (Stratagene). Relative quantitative RT–PCR was used to determine the fold difference for gene expression in comparison with basal levels in carotid arteries from uninjured rats. The real-time PCR efficiency was calculated for each primer pair using a dilution series and MJ Opticon II analysis software. Real-time PCR results were verified also by semi-quantitative RT–PCR using the ChemiDoc associated software Quantity One (Bio-Rad Laboratories) for densitometric analysis of PCR products after electrophoresis.

### Western blot analysis

Rat carotid artery segments were harvested at 3 and 7 days after arteriotomy (n = 3 for each group) and from uninjured rats (n = 3). Single carotid arteries were rinsed thoroughly with ice-cold PBS to remove blood...

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**Table 1** Summary of RT–PCR primer sequences, position, annealing temperature, PCR product length and function of the DNA repair pathway target genes

<table>
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<th>DNA repair pathway</th>
<th>Gene</th>
<th>Primer position</th>
<th>Primer sequence</th>
<th>Annealing temperature (°C)</th>
<th>PCR product length (bp)</th>
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<td>108</td>
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<td>104</td>
<td>Nuclease activity</td>
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components and were frozen immediately in liquid nitrogen. The frozen tissue was disrupted and lysed in a buffer containing 50 mmol/l Tris/HCl (pH 7.4), 250 mmol/l NaCl, 0.1 % Triton X-100 and a protease inhibitor cocktail (Roche).

The lysates were then centrifuged for 10 min at 10 000 g at 4 °C. The protein concentration in the supernatants was assessed using the Bradford assay. A portion (20 μg) of each sample was loaded, electrophoresed by SDS/PAGE on a polyacrylamide gel and electroblotted on to a nitrocellulose membrane.

Primary antibodies to detect CAT (monoclonal anti-mouse diluted 1:2000; Cell Signaling Technologies), Mn-SOD (polyclonal anti-rabbit diluted 1:1000; Upstate), p53 (monoclonal anti-mouse diluted 1:500; Santa Cruz Biotechnology) and α-tubulin (monoclonal anti-mouse diluted 1:500; Santa Cruz Biotechnology) were incubated overnight at 4 °C. All of the antibodies were used according to the manufacturers’ instructions. Immunoreactive signals were detected with an HRP (horseradish peroxidase)-conjugated secondary antibody (Santa Cruz Biotechnology) and reacted with SuperSignal (horseradish peroxidase)-conjugated secondary antibody (Santa Cruz Biotechnology) and reacted with SuperSignal WestPico or WestFemto (Pierce). Signal acquisition, quantitative analysis and normalization with respect to α-tubulin were done using the ChemiDoc-associated software Quantity One.

Histological analysis
Carotid arteries were harvested at 4 h, and 3 days and 7 days after arteriotomy for morphological and immunohistochemical analysis. Harvested vessels were fixed in 4 % buffered formaldehyde, dehydrated and embedded in paraffin. For each injured carotid artery, at least 60 serial cross-sections were observed under a light microscope at ×20 magnification; image screening and photography were performed using a Leica IM 1000 System. The carotid artery cross-sections at the injury site showing maximal remodelling and proliferative phenomena were identified and analysed further.

Cross-sections (5 μm) were stained with haematoxylin and orcein for nucleus and elastic fibre staining respectively. Image screening and photography of serial cross-sections subjected to colorimetric immunohistochemical analysis were performed using a Leica IM1000 System. Image screening and photography of serial cross-sections subjected to fluorescence immunohistochemical analysis were performed using Leica 4000F software. We considered for quantitative analysis, after all immunohistochemistry experiments, only positive cells located in the endothelium and tunica media, as the adventitia was difficult to distinguish from perivascular tissue in injured carotid arteries and could have been partially damaged during carotid artery harvesting or histological processing.

8-Oxo-dG immunohistochemical detection
Immunohistochemistry was performed on carotid arteries taken at 3 and 7 days after arteriotomy, on abdominal aorta taken from rats at 3 days after carotid arteriotomy and on control carotid arteries taken from uninjured rats immediately after anaesthesia (n = 4 for each group). The 4 % formaldehyde-fixed carotid artery cross-sections (5 μm) were deparaffinized, rehydrated and treated with Proteinase K (Roche) for 15 min at 37 °C. After washing, sections were treated with 100 μg/ml RNase A (Fermentas) for 1 h at 37 °C to increase sensitivity and specificity for DNA oxidation. DNA was then denatured with 2 M HCl for 5 min at room temperature (23 °C). After neutralization, the sections were incubated in blocking solution containing 10 % (v/v) goat serum for 1 h at room temperature and then with the primary antibody for 8-oxo-dG (monoclonal anti-mouse diluted 1:250; Trevigen) overnight at 4 °C. After washing, sections were incubated with FITC-conjugated secondary antibody (anti-mouse diluted 1:100; Jackson ImmunoResearch) for 1 h at room temperature. After washing, DNA was stained with fluorescent dye Hoechst 33258 (Sigma–Aldrich) for nuclear identification. Specimen image screening and photography were performed using a Leica F4000 System. A total of five cross-sections localized at the injury site were analysed for each carotid. The percentages of 8-oxo-dG-positive nuclei in the tunicae intima and media were calculated by determining the number of Hoechst-stained nuclei positive for 8-oxo-dG staining.

H2A.X immunohistochemical detection
Immunohistochemistry was performed on carotid arteries taken at 4 h, and 3 and 7 days after arteriotomy and on carotid arteries from uninjured rats (n = 4 for each group). The 4 % formaldehyde-fixed carotid artery cross-sections (5 μm) were deparaffinized and rehydrated. Antigen retrieval was done in a microwave through incubation in 10 mmol/l citrate buffer (pH 6). Endogenous peroxidases were blocked with 4 % (v/v) H2O2. Blocking was done in 5 % (v/v) donkey serum, followed by incubation with the primary antibody for phospho-H2A.X (polyclonal anti-rabbit diluted 1:50; Cell Signaling Technology) at 4 °C overnight. After washing, slides were incubated with biotin-conjugated secondary antibody (diluted 1:200; Santa Cruz Biotechnology). Staining was done via incubation with peroxidase–streptavidin (Vector Laboratories) for 30 min at room temperature, followed by incubation with diaminobenzidine (Vector Laboratories). Nuclei were counterstained with Mayer’s haematoxylin (Sigma–Aldrich). Image screening and photography of serial cross-sections subjected to colorimetric immunohistochemical analysis were performed using a Leica IM1000 System.
**BrdU (bromodeoxyuridine) proliferation assay**

Rats were subjected to carotid arteriotomy and, 18 h prior to killing at 3 and 7 days after injury, 50 mg of BrdU/kg of body weight was administered intraperitoneally (Roche) for detection and quantification of cells that entered into the cell cycle within 24 h of killing. Endogenous peroxidases were blocked with 4 % H₂O₂. DNA was then denatured with 2 mol/l HCl for 1 h at 37°C. After neutralization with 100 mmol/l sodium borate (pH 8.5), the sections were incubated with 0.1 % trypsin for 20 min at 37°C. After washing, the slides were incubated with the primary anti-BrdU antibody (monoclonal anti-mouse at 6 μg/ml; Roche) for 1 h at room temperature.

After further washing, the slides were incubated with biotin-conjugated secondary antibody (diluted 1:100; Santa Cruz Biotechnology). Staining was done through incubation with peroxidase–streptavidin (Vector Laboratories) for 30 min at room temperature, followed by incubation with diaminobenzidine (Vector Laboratories). Nuclei were counterstained with Mayer’s haematoxylin.

Image screening and photography of serial cross-sections subjected to colormetric immunohistochemical analysis were performed using a Leica IM1000 System. A total of five cross-sections localized at the injury site were analysed for each carotid artery. The percentages of BrdU-positive nuclei in the tunicae intima and media were calculated by determining the number of haematoxylin-stained nuclei positive for BrdU immunohistochemical staining.

**TUNEL (terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labelling) assay**

Assays were performed on carotid arteries taken at 3 and 7 days after arteriotomy and on control carotid arteries taken from uninjured rats after anaesthesia (n = 4 for each group). The 4 % formaldehyde-fixed sections (5 μm) were deparaffinized and rehydrated. The tissue was permeabilized with 20 μg/ml Proteinase K (Roche) for 30 min. After washing, TdT (terminal deoxynucleotidyl transferase) enzyme and TMR (tetramethylrhodamine) red-labelled nucleotides were added to the tissue sections, according to the manufacturer’s specifications (Roche). After 3'-OH end-labelling for 1 h at 37°C, the sections were washed in PBS and nuclei were counterstained with Hoechst 33258 (Sigma–Aldrich). Specimen image screening and photography were performed using a Leica F4000 System. A total of five cross-sections localized at the injury site were analysed for each carotid artery. The percentages of apoptotic nuclei in the tunicae intima and media were calculated by determining the number of Hoechst-stained nuclei positive for TUNEL staining. When observed with ×40–100 objectives, cells showing morphological features typically associated with apoptosis, as well as positive for the TUNEL reaction, were considered to be apoptotic. A number of randomly selected slides were quantified for TUNEL staining by two independent observers to assess inter-observer variation.

**Statistical analysis**

All statistical analysis was performed using GraphPad software (Prism 4.0). Values are presented as means ± S.E.M. Statistical significance was determined using two-way ANOVA, followed by Bonferroni’s multiple comparison test. Values of P < 0.05 were considered significant.

**RESULTS**

**Carotid arteriotomy induces DNA damage at the injury site**

Rat carotid arteries were harvested at 3 and 7 days after arteriotomy and from uninjured rats (n = 5 for each group), and the cross-sections at the injury site were subjected to immunohistochemistry for 8-oxo-dG, a marker of nucleic acid oxidative damage, and for phospho-H2A.X, a marker for DNA DSBs (double-strand breaks).

In the endothelium and tunica media, 15.5 and 24 % of nuclei were positive for 8-oxo-dG staining at 3 and 7 days after arteriotomy respectively (P < 0.05; Figure 1, upper panel). The nuclei positive for oxidative DNA damage were homogeneously distributed in all of the three tunica layers and in the perivascular tissue, around the injury site and the polypropylene stitch applied as the suture (Figure 1, lower panel). Some positive cells were also detected in contralateral uninjured carotid arteries and in the abdominal aorta in rats killed at 3 and 7 days after arteriotomy (results not shown), thus indicating a systemic oxidative stress occurring in the vasculature. A basal level of approx. 1.5 % of cells positive for 8-oxo-dG was detected in carotid arteries from healthy uninjured rats (Figure 1). It is well known that ROS can also induce oxidative RNA damage in cells. The hybridization signal detected for 8-oxo-dG was specific for nuclear DNA, as the carotid artery cross-sections were treated with ribonuclease during the experimental procedure.

Immunohistochemical analysis of phospho-H2A.X revealed increasing positive cells in injured carotid arteries ranging from 4 h to 7 days after arteriotomy (Figure 2B–2D). As immunostaining for phospho-H2A.X was localized mainly in the adventitia and perivascular tissue, the percentage of positive nuclei for this DNA damage marker is not reported, but only the qualitative analysis. Of interest, phospho-H2A.X-positive cells were localized mainly at the injury site, whereas cells positive for 8-oxo-dG staining were distributed...
Figure 1 Immunohistochemical analysis of the proliferation, apoptosis and DNA oxidative damage indexes at 3 and 7 days after carotid arteriotomy and in carotid arteries from uninjured rats

Upper panel. Percentage of cells positive for BrdU, the TUNEL assay and the 8-oxo-dG marker in the tunica media and intima. Lower panels. (A–C) Representative immunohistochemical staining of BrdU in uninjured carotid arteries (A) and injured rat carotid arteries at 3 (B) and 7 (C) days after arteriotomy. Haematoxylin nuclei counterstaining was used. Enlarged images represent ×100 magnification of the area enclosed in the black square (×40 magnification). (D–F) Representative immunohistochemical staining of 8-oxo-dG oxidative DNA damage in uninjured carotid arteries (D) and injured rat carotid arteries at 3 (E) and 7 (F) days after arteriotomy. Hoechst 33258 nuclei counterstaining was used (×40 magnification). (G–I) Representative immunohistochemical detection of apoptotic cells using the TUNEL assay in uninjured carotid arteries (G) and in injured rat carotid arteries at 3 (H) and 7 (I) days after arteriotomy. Hoechst 33258 nuclei counterstaining was used (×40 magnification). White arrows indicate representative cells positive for 8-oxo-dG (E) and the TUNEL assay (H and I). A, adventitia; M, media; L, lumen.

along the circumference of the injured carotid arteries. Cells positive for phospho-H2A.X immunostaining were barely detected in carotid arteries from healthy uninjured rats (Figure 2A).

Carotid arteriotomy induces transient apoptosis at the injury site

Rat carotid arteries were harvested at 3 and 7 days after arteriotomy and from uninjured rats (n = 5 for each...
Carotid arteriotomy induces cell proliferation in the three tunica layers

Rat carotid arteries were harvested at 3 and 7 days after arteriotomy and from uninjured rats (n = 5 for each group), and the cross-sections at the injury site were subjected to BrdU immunohistochemical detection for assessment of the cell proliferation index. At 3 days after arteriotomy, 4.4% of cells were BrdU-positive (P < 0.05), followed by an increase to 7% at 7 days (P < 0.05; Figure 1, upper panel). BrdU-positive cells were undetectable in carotid arteries from healthy uninjured rats.

BrdU-positive cells were present in both the endothelium and tunica media, as well as in focal neointima, when present (Figure 1, lower panel). BrdU-positive cells were also found in the adventitia and perivascular tissue. In particular, the presence of actively forming vasa vasorum in the adventitia at 7 days after arteriotomy was observed (Figure 1, lower panel C; ×40 magnification image).

Carotid arteriotomy affects the expression profiles of proteins involved in ROS metabolism and DNA repair/apoptosis

Rat carotid arteries were harvested at 3 and 7 days after arteriotomy and from uninjured rats (n = 3 for each group), and protein lysates were used for determining the expression level of p53, Mn-SOD and CAT. Western blot analysis revealed a significant 2.6-fold decrease in CAT at 3 days after arteriotomy (P = 0.02), followed by a return to basal levels at 7 days (Figure 3). Conversely, we did not find any significant variation in Mn-SOD after arteriotomy (Figure 3).

Finally, p53 had a significant 1.61-fold decrease at 3 days after arteriotomy (P = 0.04), followed by an increase at 7 days after vascular injury (Figure 3), but this was not statistically significant in comparison with levels detected in carotid arteries from uninjured rats, due to high variability among rats. We also analysed by Western blot the expression level of ATM (ataxia telangiectasia mutated), which is responsible for H2A.X phosphorylation, and acetylated and phosphorylated isoforms of p53 in injured carotid arteries in comparison...
with uninjured carotid arteries; however, we were unable to detect any signal, with the exception of a very faint band corresponding to acetylated p53 detectable only at 7 days after arteriotomy (results not shown). This was probably due to the very low level of expression of these proteins and to the small amount of proteins that can be extracted from small fragments of injured rat carotid arteries.

**DISCUSSION**

Arteriotomy induces the accumulation of DNA damage markers in injured carotid arteries

Experimental vascular surgical injury remains a major tool to dissect the molecular pathways involved in stenosis progression and to identify novel potential targets for (re)stenosis limitation. The pathophysiological mechanisms induced by angioplasty and arteriotomy can be different, and a model of surgical injury could be helpful in this regard. Consequently, we set up and validated an arteriotomy model of injury of the rat common carotid artery [12] that mimics the injury

The characteristics of the primer pairs used for RT–PCR and the function of the gene products are listed in Table 1. RT–PCR analysis revealed a decrease in the expression at the mRNA level of eight out of the 12 genes selected, with only two mRNAs increased (MutYh and Slk) and two genes whose mRNA level was unaffected by arteriotomy (Parp1 and Pold3).

For genes playing a role in DSB repair (Figure 4), Brca2 mRNA was expressed in uninjured carotid arteries, but was undetectable at 4 h after injury and then returned to levels at 3 and 7 days after injury. Mre11a mRNA had a transient significant 1.8-fold decrease at 4 h and 3 days after arteriotomy ($P = 0.04$), whereas the down-regulation of Xrcc4 expression by 2.7-fold was delayed until 3 and 7 days after carotid artery injury ($P = 0.01$).

A similar behaviour was detected for the expression of mRNAs of selected genes involved in MER (Figure 4). Mlh1 was expressed in uninjured carotid arteries, but was undetectable at 4 h after injury, and then returned to basal levels at 3 and 7 days after injury, whereas Prkdc had a maximal 1.85-fold decrease at 4 h after arteriotomy ($P = 0.04$), before returning to basal levels at 7 days after injury. Pold3 mRNA level was unaffected by carotid arteriotomy.

For the selected genes involved in BER, MutYh mRNA was undetectable both in carotid arteries from uninjured rats and at 4 h after arteriotomy, but it was markedly and persistently expressed at 3 and 7 days after injury. This expression profile was counterbalanced by the levels of Ogg1 mRNA, which had a transient 2.5-fold decrease at 4 h after arteriotomy ($P = 0.04$). Parp1 mRNA level was unaffected by carotid arteriotomy (Fig. 4).

Finally, for the genes included in the NER category (Figure 4), Nibhl mRNA was expressed in uninjured carotid arteries, but was undetectable at 4 h after injury and then returned to basal levels at 3 and 7 days after injury, whereas Rad23 had a 2.4-fold decrease at 3 and 7 days after arteriotomy ($P = 0.004$ and $P = 0.04$ respectively). Slk had a partially different expression profile, as its mRNA had a transient 1.8-fold increase at 4 h after arteriotomy ($P = 0.01$), followed by a persistent 1.85-fold decrease both at 3 and 7 days after arteriotomy ($P = 0.04$).

**Carotid arteriotomy differentially affects the expression profiles of DNA-repair-related genes**

Rat carotid arteries were harvested at 4 h, and at 3 and 7 days after arteriotomy and from uninjured rats ($n = 4$ for each group), and total RNAs were used for determination by RT–PCR of the expression levels of DNA-repair-related genes belonging to four distinct classes according to their mechanism of action: (i) DSB (genes analysed were Brca2, Mre11a and Xrcc4); (ii) MER (mismatch excision repair; genes analysed were Mlh1, Prkdc and Pold3); (iii) BER (base excision repair; genes analysed were MutYh, Ogg1 and Parp1); and (iv) NER (nucleotide excision repair; genes were analysed Nibhl, Rad23a and Slk).
**Figure 4** RT–PCR analysis of the expression of mRNAs coding for molecules involved in the DNA repair pathways BER, MER, NER and DSBs at 3 and 7 days after arteriotomy and in carotid arteries from uninjured rats

All measurements were normalized with respect to endogenous GAPDH levels. The values are expressed in arbitrary units as relative changes over the normalized uninjured control. Values are means ± S.E.M. For Ogg1 in BER, *P = 0.04 compared with uninjured carotid arteries; for Mre11a and Xrcc4 in DSB, *P = 0.04, #P = 0.01 and §P = 0.01 compared with uninjured carotid arteries; for Rad23a and Slk in NER, *P = 0.04 and #P = 0.01 compared with uninjured carotid arteries; for Prkdc in MER, *P = 0.04 compared with uninjured carotid arteries.

affecting arteries subjected to grafting or endarterectomy, as it is characterized by an interruption of the internal and external elastic lamina, which is considered clinically relevant for the development of arterial stenosis.

ROS formation and elimination, under physiological conditions, are well balanced. Enhanced activity of oxidant enzymes and/or decreased activity of antioxidant enzymes lead to the pathological state of oxidative stress. A previous transcriptome analysis highlighted the activation of genes involved in inflammation and ROS production in the arteriotomy model of injury [13]. On the basis of these results, we decided to analyse further the pathways involved in DNA damage and repair, as these phenomena have been well investigated in atherosclerosis and myocardial infarction, but only to a lesser extent in restenosis. The results concerning the expression of markers of oxidative DNA damage obtained in the present study are in agreement with our previous transcriptome analysis in the same model of injury [13]. We hypothesize that ROS production is increased not only by the inflammation triggered by vascular injury (namely the infiltration of monocytes and neutrophils in the injured vascular wall), but also by short-term systemic alterations of haemodynamic forces, followed by mechanotransduction in vascular cells and leading to endothelial dysfunction.

8-Oxo-dG is one of the most frequent base lesions accumulating after oxidative damage to DNA, due to the low redox potential of guanine that makes this base particularly vulnerable [14]. Oxidized guanine pairs with adenine during DNA replication, inducing G:C into T:A transversions and it is repaired primarily through the DNA BER pathway.

Phospho-H2A.X is another well-known marker of DNA damage and, in particular, of DSBs. When DSBs occur, they are always followed by the phosphorylation of H2A.X, one of the most conserved variants of the H2A protein family [15]. H2A.X is phosphorylated at Ser139 by kinases such as ATM and ATR (ATM-Rad3-related) in the PI3K (phosphoinositide 3-kinase) pathway. This newly phosphorylated protein, γ/ phospho-H2A.X, is the first step in recruiting and localizing DNA repair proteins [16].

The persistent high number of 8-oxo-dG- and phospho-H2A.X-positive cells detected in arteriotomy-injured carotid arteries and perivascular tissue in the present study possibly reflects the relevant oxidative stress induced, either directly or indirectly,
by arteriotomy, together with the lack of an effective repair of DNA damage, leading to apoptosis and cell proliferation, as demonstrated using the TUNEL assay and BrdU labelling respectively (Figures 1 and 2). The immunohistochemical findings for 8-oxo-dG are supported by the RT–PCR analysis of the Ogg1 gene (encoding 8-oxoguanine DNA glycosylase) involved in DNA BER, which had a significant decrease at 4 h after arteriotomy and was counterbalanced by the activation of the expression of Mutyh at 3 and 7 days after injury (Figure 4).

The increase in 8-oxo-dG has also been highlighted in balloon-injured rat carotid arteries [17]. The presence of phospho-H2A.X in cells in the adventitia and perivascular tissue and, to a lesser extent, in the tunica media, in carotid arteries harvested at 4 h after arteriotomy (Figure 2) is in agreement with results indicating that phospho-H2A.X is a very early marker of DNA DSBs. These results indicate the prolonged persistence after arteriotomy of DSBs, which is in agreement with the decreased expression of all of the three DSB repair genes examined by RT–PCR (Figure 4), and possibly indicates a failure of the DSB repair machinery in steps subsequent to the phosphorylation of H2A.X.

It has been hypothesized that perivascular inflammatory cells recruited after vascular injury play a role in the recruitment and/or proliferation of adventitial myofibroblasts, possibly through the release of ROS and/or cytokines, and thus contribute to vascular remodelling associated with restenosis [18]. The presence of phospho-H2A.X and of 8-oxo-dG has also been detected in atherosclerotic plaques of patients with coronary artery disease and in animal models, suggesting that DNA repair is also inefficient in this pathological state [19,20].

Overall the immunohistochemical results from the present study indicate that oxidative DNA damage, supported by the presence of 8-oxo-dG, is predominant, in reference to the number of positive cells, in comparison with DNA DSBs, supported by the presence of phospho-H2A.X (Figures 1 and 2). Moreover, our present findings also highlight a different distribution of 8-oxo-dG- and phospho-H2A.X-positive cells, with 8-oxo-dG-positive cells being homogeneously distributed in all of the three tunica layers of injured carotid arteries, and phospho-H2A.X-positive cells present mainly in the adventitia around the injury site and, to a lesser extent, in the media and neointima, when present.

Arteriotomy induces cell proliferation and apoptosis in injured carotid arteries

Arteriotomy induces a proliferative reparative reaction of the vascular wall, together with cell migration and ECM synthesis. Our present findings highlight an increase in the number of proliferating cells ranging from 3 to 7 days after arteriotomy (Figure 1). The percentage of proliferating cells assessed through the BrdU labelling in animal models of vascular injury is fairly variable, depending on the kind of injury, the target vessel and the timing of the analysis. A direct comparison of the proliferation index in the arteriotomy model with results obtained in angioplasty models could be not correct, as the arteriotomy model of injury we used in the present study induces a stenosis mainly related to a marked inward remodelling and to the formation of neovasculature, rather than to neointima as commonly observed after angioplasty [12].

DNA damage is known to block the cell cycle to allow the repair of DNA molecules. Our present results show that arteriotomy induces both DNA damage and cell proliferation, possibly indicating a failure of mechanisms leading to growth arrest [e.g. through p21, GADD45 (growth-arrest and DNA-damage-inducible protein 45), p16 and p53].

Apoptosis is an essential component of normal development as well as of most developmental abnormalities and diseases. SMC and EC apoptosis occurs after vessel injury, in remodelling and in advanced atherosclerotic lesions. Apoptosis induced by exacerbated oxidative stress prevalent under pathophysiological conditions, such as surgically induced stenosis, may profoundly affect this vascular disease. It usually occurs within a few hours or days after injury, mainly in the media and/or in the neointima, depending on the kind of injury and on the presence of atherosclerotic lesions. Vasoactive substances, such as NO, AngII and ET-1 (endothelin-1), that are often altered in injured vessels are among the regulators of apoptosis [21].

It is well known that apoptosis is stimulated by balloon angioplasty [22] and that the degree of cell loss is directly proportional to the intensity of the injury [23]. Apoptosis and proliferation are intimately coupled. The timing and level of cell apoptosis after vascular injury has been investigated using the TUNEL assay in many different animal models and vessels, generating a large amount of heterogeneous data. For example, 70% of apoptotic nuclei have been detected in carotid artery media 30 min after angioplasty in rat [24]. Other studies have revealed that apoptosis occurs in neointima from 7 to 30 days after angioplasty [25]. Furthermore, some authors have clearly demonstrated the relevant influence of the type of vascular trauma both on apoptosis and cell proliferation [26]. This last observation implies that the results from our TUNEL assay can only be partially compared with other published findings, as our surgical injury model applied to the rat carotid artery is radically different from angioplasty and could induce a different apoptotic reaction. In addition, we cannot exclude that necrotic cell death could also occur in arteriotomy-injured vessels, as hypothesized in other models of deep vascular injury [27]. Lastly, we also successfully detected apoptotic nuclei in...
the carotid artery intima (Figure 1, lower panel), whereas investigations based on balloon angioplasty were unable to report the amount of TUNEL-positive nuclei in this layer, as it is damaged and removed by the balloon [27a].

An efficient DNA repair can prevent or reduce accumulated DNA damage, thus preventing apoptosis. Consequently, the results suggesting a failure of the DNA repair machinery can be related, at least in part, to the apoptosis highlighted in the present study after arteriotomy. The higher apoptotic index detected at 3 days after arteriotomy could appear in contrast with the contemporaneous decrease in p53 (Figure 3). Nonetheless, it has been demonstrated that p53-mediated apoptosis can occur without de novo RNA and protein synthesis in some cell types [11a]. Moreover, it has been suggested that endogenous p53 can exert an anti-apoptotic action, probably related to its DNA repair activity [28].

DNA damage can induce cell apoptosis and thus be directly related to the apoptotic index reported in the present study. In this regard, it has been demonstrated recently that the presence of 8-oxo-dG in cells can activate cell death [29]. However, it should also be mentioned that the accumulation of phospho-H2A.X-positive cells could be a direct marker not only of DNA damage, but also of initial apoptosis, as it has been demonstrated that H2A.X is phosphorylated during apoptotic DNA fragmentation [30,31].

**Arteriotomy induces a transient down-regulation of p53 and CAT proteins**

The tumour-suppressor gene p53 encodes a transcription factor that activates genes involved in growth arrest and apoptosis. It has been demonstrated that adenoviral expression of p53 reduces cell proliferation in the rat carotid artery [32] or migration in the human saphenous vein [33] and, conversely, antisense oligonucleotides to p53 increase proliferation [34]. An increase in the expression and phosphorylation of p53 has been associated with markers of DNA damage and activation of DNA repair pathways, suggesting that p53 expression is ultimately triggered by DNA damage to vessel-wall cells [20], p53 can also catalyse by itself the repair of many forms of DNA damage [35,36], preventing the propagation of damaged DNA that leads to apoptosis. The temporary decrease in p53 at 3 days after arteriotomy is thus in agreement with the decrease in other DNA-repair-related genes and could be related to the activation of cell proliferation stimulated by arteriotomy (Figure 3).

CAT and SOD are antioxidant enzymes contributing to the decrease in ROS [37]. The level of superoxide anions has been demonstrated to increase in the arteries 3 days after angioplasty [38]. The decrease in CAT at 3 days after arteriotomy (Figure 3) could be responsible for an increase in H2O2 in cells in the vascular wall subjected to arteriotomy and thus contribute to the accumulation of oxidative DNA damage, as well as to cell proliferation, since H2O2 is also endowed with a potent mitogen effect [39].

**Arteriotomy affects the time-dependent expression of a panel of DNA-repair-related genes**

In mammalian cells, a network of biochemical pathways exists to maintain the functional and structural integrities of the genome. The decrease in the majority of DNA-repair-related genes analysed in the present study indicates at least a partially compromised DNA repair machinery in the acute phase following arteriotomy. To our knowledge, the present study is the first that describes in detail the expression profile of a dataset of genes involved in DNA repair after vascular injury. Nonetheless, it should be underlined that this subgroup of genes is only representative of a complex mechanism that is based on the contribution of a number of factors regulated at different levels.

A previous study has demonstrated the effectiveness of the local delivery of RAD50, a gene playing a key role in recognizing and signalling DSBs, in reducing intimal hyperplasia in a model of porcine coronary stent deployment [40]. These findings are of interest as they indicate that an overexpression of proteins involved in DNA repair could be helpful in the regression of restenosis.

**Conclusions**

To our knowledge, the present study is the first time-dependent expression analysis of an extended panel of DNA-repair-related genes, together with the immunohistochemical analysis of DNA damage markers and the determination of cell proliferation and apoptosis indexes conducted in parallel in the same model of surgical vascular injury.

Persistent DNA damage may negatively affect a variety of cellular processes in vascular stenosis induced by surgical injury, including cell survival and proliferation. Antioxidant therapy could be helpful in reducing the harmful effects of oxidative stress on the vascular wall subjected to injury, as demonstrated by studies conducted in patients, animal models and *in vitro* [41–46].

Our findings are descriptive and further analyses are currently in progress to dissect their underlying mechanisms. Nonetheless, these results provide information on the molecular events triggered by vascular surgical injury and suggest that molecules involved in DNA damage and repair could represent a good target for reducing restenosis, and that the best therapeutic window for modulation of these molecules should be carefully considered on the basis of their expression profiles and in the context of the vascular injury applied.
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