



Purinergic receptor distribution in endothelial cells in blood vessels: a basis for selection of coronary artery grafts

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Abstract

Expression levels of the purinergic P2X receptor subunits (P2X₁ to P2X₇) and P2Y₂ were examined in the endothelial cell layer of internal mammary artery (Ann. Thorac. Surg. 54 (1992) 652), radial artery (Ann. Thorac. Surg. 16 (1973) 111) and saphenous vein (Ann. Thorac. Surg. 20 (1975) 628) samples obtained at surgery for coronary artery bypass grafts using immunohistochemistry and confocal microscopy. Similar levels of P2X₁, P2X₂, P2X₃, P2X₇, and P2Y₂ were found in the endothelial cells in all vessels examined while the levels of P2X₅ and P2X₆ were uniformly lower. A clear difference was measured in P2X₄ expression between arteries and veins. Both radial and internal mammary arteries exhibited very low levels of P2X₄ whereas the level in the saphenous vein was 14.6 fold higher ($P < 0.0001$), approaching that of the major receptor subtypes. These data showing strong expression of P2X₄ in veins have implications for the choice of vessels used in coronary artery bypass grafts given that P2X₄ is involved in calcium influx into endothelial cells, modulates blood vessel contractility and is up-regulated in situations involving intima proliferation suggesting vein grafts are more susceptible to developing atherosclerosis. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

The left internal mammary artery (LIMA) is currently the conduit of choice for coronary artery bypass [1] due to better survival and freedom from all cardiac events, an improved exercise tolerance and increased longevity of patency of the vessel compared with the saphenous vein (SV). The LIMA shows a patency of 85–90% 10 years after implantation, compared with approximately 50% for the SV [1–4]. The evident superiority of the LIMA in coronary artery bypass surgery has resulted in interest in other arterial conduits as an alternative or adjunct to LIMA and saphenous vein, including the right internal mammary artery (RIMA), radial artery (RA), gastroepiploic artery and inferior epigastric artery.

The RA was initially used as a conduit in the 1970s

[5] but was discontinued due to high rates of graft failure [6,7]. It was reintroduced by the same group in 1992 [8], with improved harvesting techniques and the use of antispasmodic drugs leading to excellent short-term results of 92% patency at 1 year. Although 10 year patency rates are not yet available, the 5 year patency rate is reported as about 85% for the RA [9,10] compared with 91% for the LIMA [9] and 55% for the SV [4]. Differences in patency between RA and LIMA may be related to the graft implantation sites as the LIMA is usually anastomosed to the larger LAD, while the RA tends to be used for the circumflex system and right coronary artery [9]. When used as a free graft, the IMA has shown a 5 year patency rate of 84% [11].

Differences in late patency rates between vein and artery have largely been ascribed to the development of the so-called saphenous vein graft disease that leaves patients at a progressively increased risk of ischemic complications [12]. Saphenous vein grafts develop intimal hyperplasia [13] and demonstrate accelerated

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atherosclerosis from 5 to 10 years [2,14], whereas the IMA at 10 years shows little evidence of atherosclerosis.

Purinergic receptors of type P2X are fast-acting ionotropic channels largely selective for calcium. There are seven identified subtypes (P2X₁–P2X₇) and of these the P2X₄ receptor is present in the vascular system of most organs and so is predicted to play a role in the modulation of blood vessel contractility [15]. ATP is released as a co-transmitter with noradrenaline and has been shown to mediate sympathetic vasoconstriction in small arteries and arterioles [16] and to regulate pulmonary arterial tone [17]. A rabbit model of atherosclerosis in which the aorta developed a greatly expanded neointima following balloon injury and cholesterol feeding was used to examine factors associated with the condition [18]. In particular, the redistribution of purinergic P2X receptor subunits was examined. While most receptors remained unaltered, P2X₄ clusters were found in much higher density throughout the media and in very high density in the enlarged neointima following balloon injury. In particular, receptor density on endothelial cells increased about 10-fold after balloon injury. All P2X receptor subtypes were found on endothelial cells. These data suggested the release of ATP from damaged endothelial cells as well as from smooth muscle cells is sufficient to activate P2X₄ receptors and that these may be associated with neointimal proliferation.

In this study, we examined the distribution of P2X₁–P2X₇ and describe for the first time the distribution of the metabotropic purinergic P2Y₂ receptors in the endothelial cell layer of human internal mammary artery, radial artery and saphenous vein using a new subtype-specific antibody. P2Y₂ receptors have long been known to play a role in endothelial cells [19,20]. These vessels were obtained at surgery for coronary artery bypass grafts, enabling comparisons to be made of the intrinsic levels of P2 receptor expression present in each as a measure of their suitability for resisting atherosclerosis.

2. Methods

2.1. Production of P2Y₂ antiserum

The consensus sequence of the human P2Y₂ [21] cloned receptor was examined for suitable epitopes following the successful production of antibodies to rat P2X_{1–7} subtypes [22,23] and human P2X_{1–7} subtypes [24]. The intracellular C-terminal epitope corresponding to the segment Leu226-Lys242 was utilized after attaching a C-terminal Cys residue for cross-linking to diphtheria toxin using maleimidocaproyl-*N*-hydroxysuccinimide. All syntheses were carried out using standard *t*-BOC chemistry on an ABI synthesiser [25]. The pep-

tide-antigen conjugate was suspended in water at 5 mg/ml and aliquots emulsified by mixing with Complete Freund's Adjuvant. Emulsion volumes of 1 ml containing 2 mg of peptide epitope were injected intramuscularly with second, third, fourth and fifth immunisations followed at 2 week intervals using Incomplete Freund's Adjuvant. Final bleeds via venepuncture were obtained at 10–12 weeks, after it was established that adequate antibody titers had been obtained in the rabbits. The blood was incubated at 37 °C for 30 min, and stored at 4 °C for 15 h after which the serum was collected following centrifugation and stored at –20 °C in small aliquots. Sera were tested with an ELISA assay. The antibody titer, defined as the reciprocal of the serum dilution resulting in an absorbance of 1.0 above background in the ELISA assay, was in the range 75 000 ± 4000 compared with 225 ± 25 for pre-immune serum.

2.2. Antibody specificity

P2Y₂ antibody bound selectively and specifically as shown by Western blots using vascular smooth muscle cell homogenates. Cells were trypsinized and washed with phosphate buffered saline (PBS) and cells in suspension were transferred into falcon tubes. Cells were then centrifuged for 10 min at 4000 rpm, the supernatant was decanted and pellets placed on ice. Pellets were resuspended in 1 ml of PBS and centrifuged for 5 min at 10 000 rpm. Supernatants were decanted and pellets solubilized in PBS/0.1% Triton in the presence of protease inhibitors. Cell suspensions were then centrifuged at 14 000 rpm for 10 min at 4 °C, supernatants were removed, 6 × SDS gel sample buffer added for 5 min at 100 °C to denature the proteins. Standard SDS-PAGE procedures according to the method of Laemmli [26] were undertaken using a NOVEX X-cell II mini system to run 10–20% gradient gels. Sample protein loadings were 0.16 mg, as determined by a Dc Biorad assay, in PBS containing protease inhibitors. Cell supernatant (15 µl) in SDS gel sample buffer was loaded. Gels were stained for 1 h using 0.5% Coomassie Brilliant Blue R-250 in 40% methanol/10% acetic acid and destained in 40% methanol/10% acetic acid.

Proteins were transferred from the gels to nitrocellulose membranes (Amersham) using a NOVEX X Cell II Blot Module and transfer buffer (2.9 g glycine, 5.8 g tris, 0.37 g SDS, 20% methanol in 1 l). Membranes were blocked with PBS/2% skim milk powder overnight and rinsed with PBS/0.1% Tween/2% skim milk powder. Rabbit pre-immune serum and the primary P2Y₂ antibody were used at a 1:500 concentration and anti-rabbit HRP was used at a 1:2000 concentration. Primary antibody was incubated for 1 h and secondary antibody for 30 min. Detection of antibody bands was achieved using the ECL/Hyperfilm system (Amersham).

2.3. Tissue preparation and immunohistochemistry

All tissue collection was undertaken after informed consent in accordance with protocols approved by the local hospital ethical committee. Samples of vessels used in coronary artery bypass grafts were fixed in 4% paraformaldehyde in PBS pH 7.2 for 12 h immediately after harvesting and then cryoprotected in 30% sucrose in PBS pH 7.2 for 24 h prior to transverse sections being cut on a freezing microtome (Reichert Jung 2800 Frigocut).

Indirect immunohistochemical and immunofluorescent methods were used. Cy 3 conjugate of donkey anti-rabbit secondary antibody, adsorbed against con-specific IgGs, was purchased from Jackson Immuno-research (West Grove, PA). All other reagents were purchased from Sigma Chemical Co (St. Louis, MO). Specific antibodies against the extracellular domains of all P2X subtypes were produced in the laboratory as described [22,23,27,28]. Sections (30 μ m) of fresh tissue were placed in 0.1% dimethyl sulphoxide (DMSO) in phosphate buffered horse serum (100 ml PBS, 2 ml normal horse serum (NHS), 0.1 ml Triton X-100, 1 g bovine serum albumin (BSA) for 30 min and then washed in PBS for 3 \times 10 min and immersed in 20% NHS in PBS for 1 h to block non-specific binding sites in the tissue. This was followed by incubation with 1:100 of primary antibody in PBS for 12 h at 20 $^{\circ}$ C. Slides were rinsed in PBS (3 \times 10 min) followed by secondary fluorescent antibody for 90 min at 20 $^{\circ}$ C. Slides were then washed in PBS (3 \times 10 min), cover-slipped and sealed. All sections were viewed on a Leica TCS NT UV laser confocal microscope system with the pin hole set at 1.0 as a compromise between depth of focus and background labeling. Specific binding of antisera was checked using the appropriate blocking peptides consisting of 10 μ M of the peptide epitope added prior to the antiserum. No binding of antiserum was evident. As an alternative, unfixed frozen tissue was cut on the cryotome and sections were fixed overnight in acetone at -20 $^{\circ}$ C. Sections were then

air-dried and incubated in 1% H₂O₂/BSA in PBS prior to washing (3 \times 5 min) to remove endogenous peroxidase activity. Horse serum (5%) was added for 15 min to block non-specific binding and the slides were incubated in primary antibody at 1:200 for 2 h at room temperature. After washing, sections were incubated in HRP-conjugated goat anti-rabbit IgG (Dako) at 1:100 with PBS. Label was visualized with 0.1% diaminobenzidine tetrahydrochloride (DAB, Sigma) for 10 min. Control slides were treated with pre-immune serum or preincubated with the cognate peptide resulting in the complete blocking of labeling showing very good binding specificity.

2.4. Statistical evaluations

Each frozen patient sample was sectioned three times for each subtype of antibody label and confocal images collected from at least three areas of endothelium from each section under strictly identical conditions. Each tiff image was analyzed using NIH image with at least three determinations made of endothelial label intensity. Averages were then obtained for each patient. Each measurement finally recorded was the mean of at least nine determinations for each patient sample with each label. Statistical evaluation was performed using the nonparametric Wilcoxon two-tailed test with significance if $P < 0.05$ with n being the number of patients, not the much higher number of separate measurements for each patient.

3. Experimental results

3.1. Distribution of P2Y₂ in VSMC and endothelial cells

A single clear major band was detected in Western blots corresponding to glycosylated P2Y₂ at approximately 53 kDa, midway between 42 and 60 kDa protein standards (Fig. 1). Specificity of the antibody was also shown by discrete immunohistochemical labeling of VSMC in which the P2Y₂ appeared to label in small puncta within the cells and outside the nucleus (Fig. 2a). This labeling was blocked using preabsorption with 10 μ M of the cognate peptide (Fig. 2b) further demonstrating specificity. Replacement of P2Y₂ antibody (or any of the P2X antibodies) with pre-immune serum similarly resulted in the complete loss of staining.

3.2. P2X₁₋₇ and P2Y₂ receptor expression in intact endothelium in vein and arteries

Each of the patient samples of IMA, RA and SV were labeled with each of the subtypes P2X₁–P2X₇ and P2Y₂ and 25 μ m fields collected from three to four

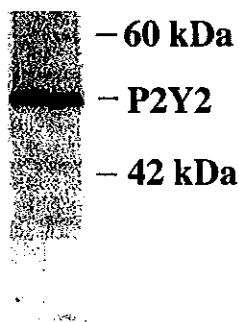


Fig. 1. A Western blot of P2Y₂ in VSMC homogenate showing a single major band at about 53 kDa corresponding to glycosylated receptor.

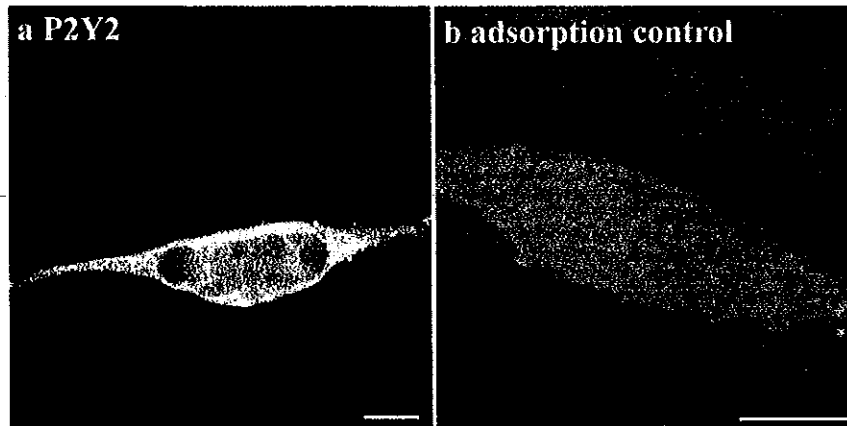


Fig. 2. Labeling of fixed and permeabilized VSMC with P2Y₂ antibody in the absence (a) and presence (b) of 10 μM blocking peptide. Protein is labeled outside the nucleus, mostly on the cell membrane in the absence of blocking peptide.

areas from each of three sections of vessel. Fig. 3 shows DAB stained sections of the three types of vessels used in the study. On the left are vessels stained for P2Y₂ while those on the right are stained for P2X₄. The top panels (a, b) show IMA, the next pair (c, d) show RA and the lower pair (e, f) SV. While only the SV exhibits P2X₄ label on the endothelium (Fig. 3f), all three vessels exhibit P2Y₂ label on the endothelium. Fig. 4 shows examples of SV sections labeled with seven different antibodies and a control, recorded using confocal microscopy. The panels were each obtained under identical conditions, making direct comparisons of fluorescence intensity possible in the linear region of NIH image analysis. Almost identical results were obtained for each of the arteries so that mean intensities were largely indistinguishable between any of the vessels for these subtypes with the possible exception of P2X₆. The intensity of P2X₂ label was consistently higher than the other subtypes with P2X₅ and P2X₆ substantially lower and the remaining subtypes intermediate. The situation with P2X₄ label is shown separately in Fig. 5. Examples of typical labeling are shown for arteries as represented in Fig. 5a with an IMA, in which negligible P2X₄ is found on the endothelium. In contrast, SV samples (Fig. 5b) showed that quite high levels are present. Data were collected for a total of 8 IMA, 5 RA and 6 SV with each patient sample labeled in three separate sections and each section sampled at least three times. The results are shown in Table 1. The average intensities are listed together with the S.D. for each antibody label (n = patient number). The results show good consistency in all vessels with the exception of P2X₄ and to a far lesser extent P2X₆. A statistically significant difference ($P < 0.014$) was found when comparing RA and SV with P2X₆ using the Mann–Whitney test, however, the primary difference was obtained with P2X₄ in SV

compared with either artery where the difference was an extremely significant $P < 0.0001$.

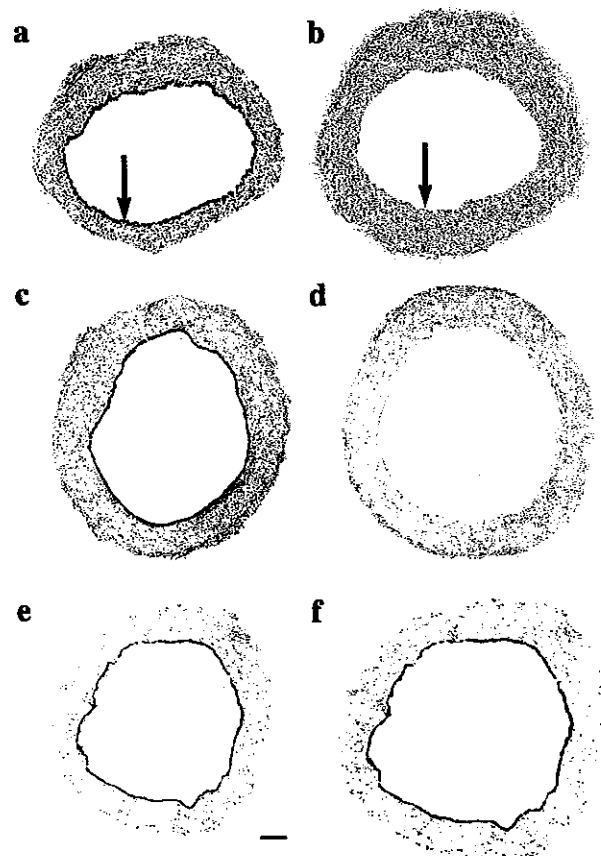


Fig. 3. DAB stained IMA (a, b), RA (c, d) and SV (e, f) for P2Y₂ (a, c, e) and P2X₄ (b, d, f). The endothelium (arrows in a, b) show label for P2Y₂ (similar in all vessels) but not for P2X₄ in IMA and RA. Bar = 200 μm.

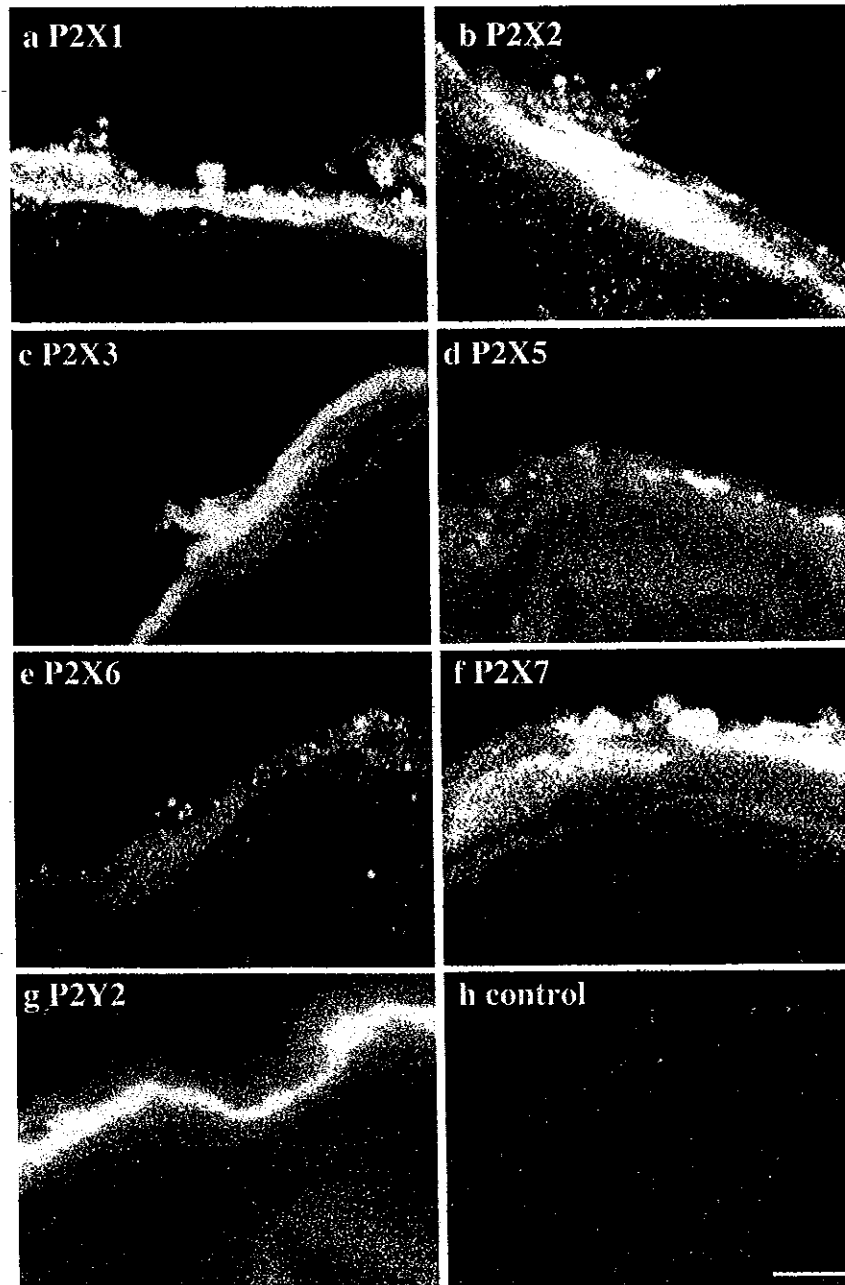


Fig. 4. Frozen section of the endothelial surface of SV recorder under identical conditions with confocal microscopy and labeled for the different receptors shown. P2X₁, 2, 3, 7 and P2Y₂ all label heavily while P2X₅, 6 exhibit much lower abundance. The control panel shows P2Y₂ labeling in the presence of 10 μ M of the epitope peptide is abolished. Identical results are obtained with IMA and RA. Bar = 5 μ m.

4. Discussion

We have been able to directly monitor the expression of human P2Y₂ in the endothelium of blood vessels for the first time by using our newly described specific P2Y₂ antibody. P2Y₂ expression increases in balloon injured aorta endothelial cells and neointimal cells, especially those at the luminal surface. Moreover, ATP, acting through P2Y₂ receptors, appears to be involved in the smooth muscle cell hyperplasia observed in the damaged intima. P2Y₂ may also be involved in the chronic

constriction at the site of lesion and the consequent restenotic process [29]. However, we have found no discernible differences in endothelial cell expression of P2Y₂ in the different vessel grafts.

The P2X₄ receptor has similarly been implicated in the modulation of blood vessel contractility [15]. Direct identification of P2X₁ and P2X₂ in the endothelium of several different rat blood vessels was demonstrated by Hansen et al [30] but no quantitative comparisons between P2X subtype binding were determined. Our later investigation of the role of the P2X receptors in

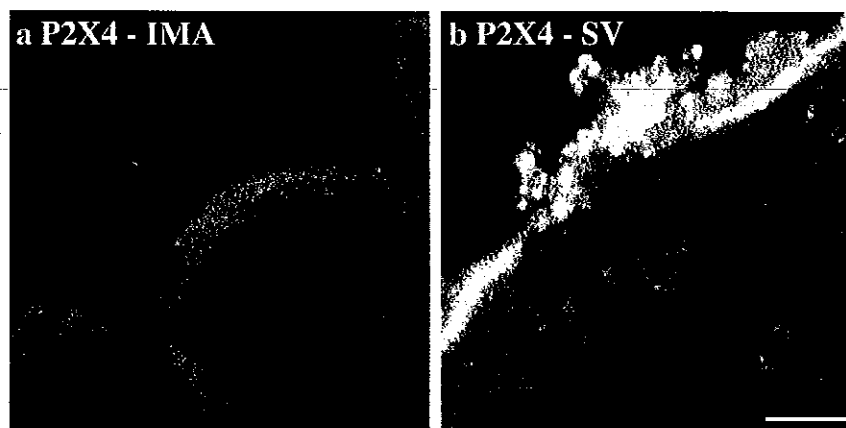


Fig. 5. Frozen section of IMA (a) and SV (b) each labeled with P2X₄ antibody showing that the endothelium in arteries is essentially devoid of P2X₄ while abundant in SV. Bar = 5 µm.

Table 1

Mean (± S.D.) intensity of endothelial cell label for each of the antibodies under identical conditions

Vessel	P2X ₁	P2X ₂	P2X ₃	P2X ₄	P2X ₅	P2X ₆	P2X ₇	P2Y ₂
IMA	153 ± 47	205 ± 17	144 ± 13	8 ± 2	89 ± 14	57 ± 16	188 ± 12	156 ± 23
RA	156 ± 37	207 ± 29	152 ± 41	11 ± 2	68 ± 14	37 ± 17	164 ± 32	156 ± 30
SV	158 ± 27	212 ± 25	171 ± 16	139 ± 16	76 ± 15	73 ± 15	180 ± 11	124 ± 30

A Wilcoxon two-tailed *t*-test shows that P2X₄ labeling intensity in SV differs significantly from IMA and RA ($P < 0.0001$).

neointimal proliferation has shown that while expression levels of most receptors remain unaltered, P2X₄ receptor density increases about 10-fold on endothelial cells and underlying neointimal cells after balloon injury in the rabbit aorta [18] with the level in the normal aorta being to the levels found in the IMA and RA specimens in the current study. Thus, P2X₄ and P2Y₂ receptors in the endothelium both appear to be significantly affected by balloon injury but only P2X₄ expression differs between the different graft types.

In all three types of coronary artery graft vessels examined, the level of P2Y₂ appears little different whereas levels of P2X₄ are very significantly lower in IMA and RA vessels but uniformly high in the SV samples. This suggests that P2X₄ may play a more significant role in arteriosclerosis and restenosis than P2Y₂. Furthermore, the demonstrated pre-existing high levels of P2X₄ in SV may be a principal reason why these vessels are found to be clearly more susceptible to arteriosclerosis and exhibit a significant reduction in graft patency longevity. We conclude from these results that where vessel choice is readily available and maximum graft lifetime is sought, venous grafts should be avoided due to their underlying susceptibility to P2X₄-associated intima proliferation.

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