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REVIEW

P2 receptors: new potential players in atherosclerosis

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Atherosclerosis is a focal inflammatory disease of the arterial wall. It starts with the formation of fatty streaks on the arterial wall that evolve to form a raised plaque made of smooth muscle cells (SMCs), and infiltrating leukocytes surrounding a necrotic core. The pathogenesis of the atherosclerotic lesion is incompletely understood, but it is clear that a dysfunction of the endothelium, recruitment and activation of inflammatory cells and SMC proliferation have a pivotal role. Over recent years receptors for extracellular nucleotides, the P2 receptors, have been recognized as fundamental modulators of leukocytes, platelets, SMCs and endothelial cells. P2 receptors mediate chemotaxis, cytokine secretion, NO generation, platelet aggregation and cell proliferation in response to accumulation of nucleotides into the extracellular milieu. Clinical trials have shown the benefit of antagonists of the ADP platelet receptor(s) in the prevention of vascular accidents in patients with atherosclerosis. Therefore, we anticipate that a deeper understanding of the involvement of P2 receptors in atheroma formation will open new avenues for drug design and therapeutic intervention.
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Abbreviations: ELAM, endothelium leukocyte adhesion molecules; E-NPP, ecto-nucleotide pyrophosphatase/phosphodiesterase; E-NTPDase, ecto-nucleoside triphosphate diphosphohydrolase; GPI, glycosylphosphatidyl inositol; ICAM-1, intercellular adhesion molecule-1; IP_3, inositol trisphosphate; LDL, low density lipoprotein; LPS, lipopolysaccharide; MCP-1, macrophage chemotactic protein-1; M-CSF, monocyte-colony stimulating factor; MMPs, metalloproteinases; oxLDL, oxidized low density lipoprotein; PDGF, platelet derived growth factor; PP_i, inorganic pirophosphate; P2R, P2 receptor; SMCs, smooth muscle cells; VCAM-1, vascular cell adhesion molecules-1; VLA-4, very late antigen-4

Introduction

Atherosclerosis is a leading cause of morbidity and morbility in the western world. Although it was previously thought to be mainly a degenerative disease, it is now well ascertained that its pathogenesis is inflammatory. While retention of atherogenic lipoproteins and foam cell accumulation into the arterial intima are the main morphological stigma of atherosclerosis (Stary, 1989; Libby *et al*., 1996), more subtle changes in the microenvironment of the arterial wall caused by infiltration of inflammatory cells and local release of cytokines and other inflammatory mediators are increasingly recognized as additional key factors in the initiation and progression of the atherosclerotic lesion (Dong *et al*., 1998; Gu *et al*., 1998b; Phipps, 2000). The 'inflammatory hypothesis' is not only one of the most stimulating recent advances in the understanding of atherogenesis, but also a conceptual breakthrough susceptible of far reaching therapeutical developments, given the high prevalence of inflammation as a risk factor in the population and the potential availability of preventive measures.

In the context of the 'inflammatory hypothesis' the endothelium has a central role (Figure 1). Under physiological conditions the endothelial lining is crucial for haemostasis, platelet activation, coagulation, fibrinolysis, modulation of vascular tone and smooth muscle cell proliferation (Enderle *et al*., 2000; Bryan *et al*., 2001) In response to various challenges, endothelial cells may change their functions and shift from an anti-coagulant to a pro-coagulant status, or initiate synthesis and release of vasoactive factors, cytokines or growth factors. These modifications as a whole are recognized as key features of the so called 'endothelial dysfunction' (or 'endothelial instability') that is thought to participate in plaque formation and to precipitate the most severe consequences of this lesion (Forgione *et al*., 2000; Caillaud *et al*., 2001), even in the absence of an overt rupture of the plaque.

Many factors may lead to endothelial dysfunction and to atherosclerosis, including an increased serum LDL concentration, presence of altered LDL, free radicals, hypertension, cigarette smoking, diabetes, high serum homocysteine levels, chronic infections (e.g. by *Helicobacter pylorii, Streptococcus*

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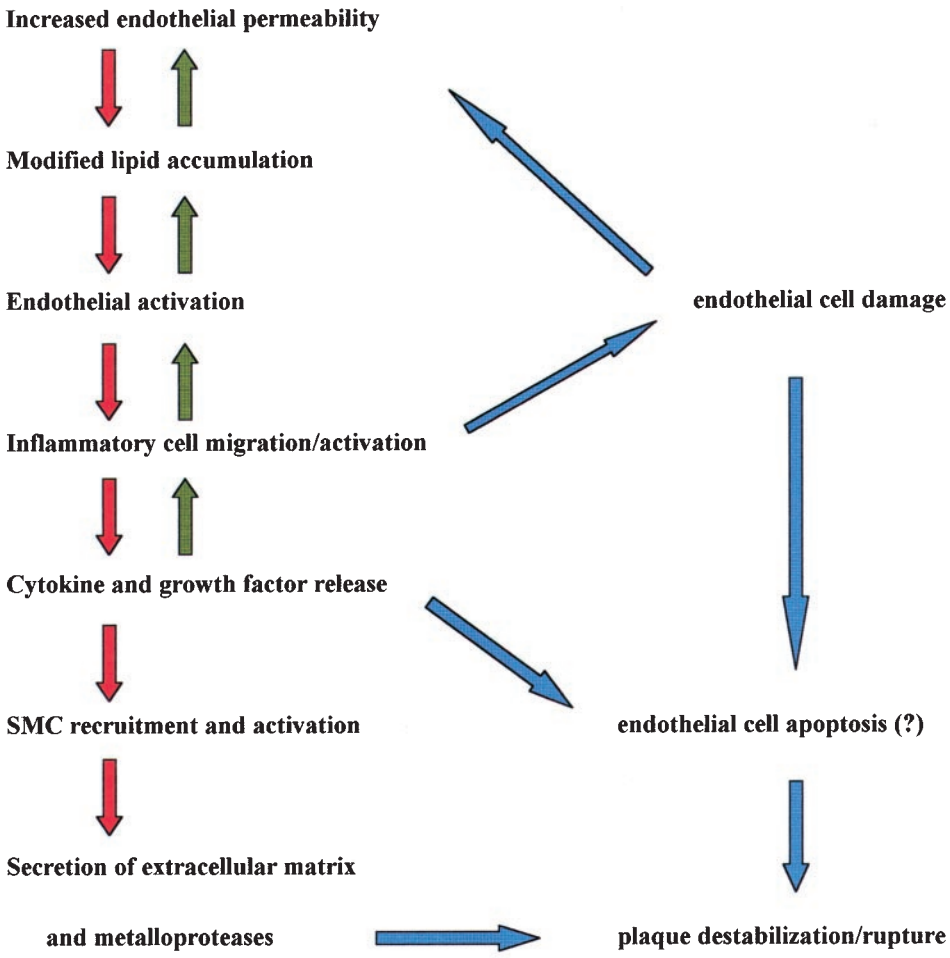

Figure 1 Steps in atheroma formation and progression.

Pneumoniae, Herpes virus), endotoxaemia, and an unfavourable genetic background (Briner & Luscher, 1994; Heitzer *et al*., 1996; Nygard *et al*., 1997; Goldboult & Neufeld, 1988; Kullo *et al*., 2000; Shah, 2001). However, local conditions at the level of the arterial wall predisposing to this outcome are poorly known.

During the last few years the plasma membrane receptors for extracellular nucleotides have been recognized as important modulators of responses of blood cells (i.e. platelets, monocytes, granulocytes, lymphocytes) as wells as of cells of the vessel wall (i.e. endothelial cells, smooth muscle cells (SMCs) and fibroblasts) (Ralevic & Burnstock, 1998; Burnstock, 1999; Burnstock & Williams, 2000; Dubyak, 2000; Di Virgilio *et al*., 2001; Lewis & Evans, 2001). These receptors, named P2 receptors (P2R), are known to have an undisputed and crucial role in the modulation of vascular tone, and to be in perspective of similar importance as regulators of the inflammatory response (Di Virgilio, 1995; Brambilla *et al*., 1999; Souslova *et al*., 2000; Solle *et al*., 2001; John *et al*., 2001). Additionally, P2 receptors have a strong cardioregulatory activity that further emphasizes their potential in cardiovascular diseases (see Vassort, 2001, for a recent comprehensive review). Since the natural ligands for the P2R, ATP and ADP (and possibly UTP/UDP), are released into the blood under many circumstances (platelet aggregation, shear-stress or damage of the endothelial lining due to infections or surgical manoeuvres), it would not be surprising that the P2R were stimulated during plaque formation and participated actively in this process.

The atherogenic process

Changes in permeability of the endothelial barrier are crucial from the very beginning of atheroma formation. Recruitment of monocytes within the intima of large size artery occurs early during atherosclerosis, reflecting a peculiar pattern of expression of endothelial adhesion molecules such as endothelium leukocyte adhesion molecules (ELAM) and vascular cell adhesion molecule-1 (VCAM-1) (Cybulsky & Gimbrone, 1991). VCAM-1 bind the specific integrin receptor very late activating antigen 4 (VLA-4) expressed by monocytes and lymphocytes, but not granulocytes, thus probably explaining the selective recruitment of monocytes in early atherogenesis (Cybulsky *et al*., 1991; Bochner *et al*., 1991). Hypercholesterolemic diet appears to cause upregulation of VCAM-1 well before fatty streak formation takes place (Li *et al*., 1993). At the same time, soluble products such as macrophage chemotactic protein-1 (MCP-1) and macrophage colony stimulating factor (M-CSF) produced by endothelial cell, SMCs and the inflammatory cells themselves under the stimulation of locally released cytokines, accumu-

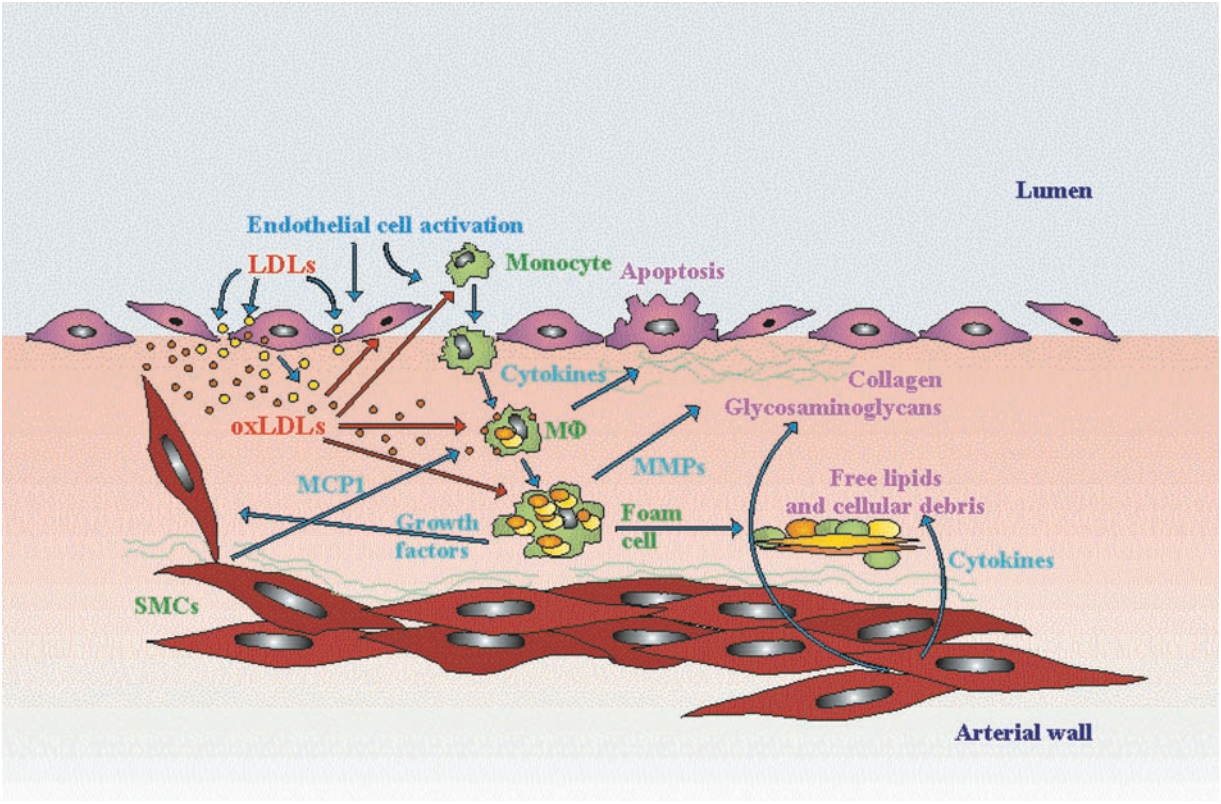

Figure 2 Events in atherosclerotic plaque formation. Injury or activation of endothelial cells allows penetration of molecules (e.g. LDL) from the arterial lumen into the subintimal space. Within this space such molecules may undergo modifications (e.g. oxidation) that unable them to further activate intimal cells, and also accelerate phagocytosis by macrophages (MΦ). Endothelial cell activation also facilitates migration of monocytes from the blood. Monocyte migration is sustained by release of chemotactic factors (e.g. MCP-1) by SMCs. Monocytes differentiate into macrophages and phagocytose oxLDLs and other extracellular lipids. During phagocytosis, macrophages become activated and release cytokines and growth factors. Lipid laden macrophages further differentiate into foam cells that eventually die, releasing their content into the necrotic core of the plaque. The plaque undergoes a continuous remodelling due on the one hand to deposition of extracellular matrix protein and on the other to secretion of MMPs that digest the extracellular matrix. Extracellular matrix proteins are secreted by SMCs. MMPs are secreted by SMCs and macrophages. SMCs under the stimulation with LDL, cytokines and growth factors actively proliferate thus forming the fibrous cap (not shown in the Figure). Some of the products released into the plaque may accelerate damage of the endothelial lining by causing apoptosis of endothelial cells.

late in the arterial wall and support recruitment as well as activation-maturation of monocytes (Figure 2).

Migration of inflammatory cells into the intima is paralleled by infiltration of low density lipoproteins (LDL), to promote deposition of fatty streaks, a typical arterial lesion made of lipid-filled foam cells, T lymphocytes and a small amount of extracellular lipids. Fatty streak formation is now considered a pure inflammatory lesion. Mechanical forces acting on the arterial wall have a key role in facilitating plasma lipoprotein influx and in causing the adaptative intimal thickening typical of atherosclerosis (Gimbrone *et al*., 2000). If the offending agents are not removed, the inflammatory response can continue indefinitely, stimulating proliferation and migration of SMCs, and leading to formation of an intermediate lesion and to remodelling of the arterial wall. Fatty streaks undergo a progressive increase with intra and extracellular lipid deposition and enhanced extracellular matrix accumulation. The number of SMCs increases, either by *in situ* intimal proliferation or as a consequence of migration from the media (Gorski & Walsh, 1995).

Changes in the endothelial surface may contribute to plaque growth in several ways. In first place, a reduced ability to produce anti-thrombotic factors (e.g. prostacylins and NO) may promote platelet aggregation and mural thrombosis (Falk & Fernández-Ortiz, 1995; Diodati *et al*., 1998; Hasenstab *et al*., 2000). Secondly, the production of heparin-like glycoaminoglycans, thrombomodulin and plasminogen may also be decreased. Platelet deposition and activation on the endothelium generates products that further promote SMCs migration and plaque growth (e.g. platelet-derived growth factor, PDGF). SMCs are crucial in the atherogenic process, therefore understanding the physiology of factors that modulate their function, motility and growth is of the utmost importance.

Under normal physiological conditions, SMCs are confined to the medial layer of the artery wall, but early in the genesis of the atherosclerotic plaque they undergo mitogenic stimulation and phenotype change, losing contractile elements and acquiring the ability to replicate and migrate into the intima (Gorski & Walsh, 1995). Within the intima SMCs proliferate, start deposition a fibrotic connective tissue matrix and undergo an 'inflammatory differentiation' that enables them to secrete pro-inflammatory agents (e.g. cytokines and metalloproteases). Secretion by SMCs of IL-8, MCP-1, TNFα, IL-1, TGFβ has been documented (Watson *et al*., 1998).

Recently, it has been emphasized that a tight paracrine interaction between endothelial cells and SMCs might condition the progression of the plaque (Rainger & Nash, 2001). Endothelial cells, under the effect of soluble factors released by the SMCs, acquire an highly reactive inflammatory phenotype that on the one hand increases leukocyte adhesion and diapedesis, and on the other sensitizes them to cytokines released into systemic circulation. This could be an important factor in the increased susceptibility to atherosclerotic occlusive disease associated with chronic infections (Kiechl *et al*., 2001).

Production of extracellular matrix is another additional feature of the atherosclerotic lesion. The fibrous cap that is thus formed owes its genesis to several factors that operate a continuous remodelling of the plaque: deposition and degradation of extracellular matrix, SMCs proliferation, lipid deposition, macrophage and lymphocyte infiltration. Stable plaques often have a relatively thick fibrous cap protecting the lipid core from contact with blood. In the vulnerable, unstable, lesion we may observe a substantial lipid core and a relatively thin cap. Within these latter lesions, pro-inflammatory agents produced by T cells (e.g. IFNγ) seem to have a key role, on the one hand by decreasing SMC secretion of extracellular matrix proteins, and on the other by increasing production by macrophage (but also by SMCs) of metallo proteinases (MMPs) (Galis *et al*., 1995; Libby *et al*., 1996).

P2 receptors in the vasculature

P2R are ubiquitously expressed throughout the human body, the vessel wall included. The P2YR are seven membrane spanning receptors coupled *via* G protein ($G_{i/o}$ or $G_{q/11}$) to IP_3 generation, Ca^{2+} release from intracellular stores or adenylate cyclase stimulation/inhibition (Cooper & Rodbell, 1979; von Kugelgen & Wetter, 2000; Communi *et al*., 2001). P2XR are membrane ion channels made by the assembly of subunits of the same (homo oligomers) or different (hetero oligomers) subtype (Table 1) (North & Surprenant, 2000; Khakh *et al*., 2001). Among P2X receptors, $P2X_7$ is endowed with the ability to generate a non-selective plasma membrane pore upon sustained stimulation by extracellular ATP and directly interacts with several cytoplasmic proteins (Di Virgilio, 1995; Kim *et al*., 2001). The principal P2R subtypes present in endothelial cells are $P2Y_1$ and $P2Y_2$, but mRNAs for $P2Y_4$ and $P2Y_6$ have also been identified (Jin *et al*., 1998). Although it is assumed that P2XR are expressed by endothelial cells to a very low level, it is likely that this belief will have to be re-evaluated as further studies are carried out. Functional evidence suggests that endothelial cells express the $P2X_7R$ (von Albertini *et al*., 1998; Goepfert *et al*., 2000), while molecular studies reveal expression of $P2X_4$ (Yamamoto *et al*., 2000; Korenaga *et al*., 2001). There are so far no reports documenting expression by endothelial cells of other members of the P2 family (i.e. $P2Y_{11}$, $P2Y_{12}$, $P2X_1$, $P2X_2$, $P2X_3$, $P2X_5$ and $P2X_6$).

Vascular smooth muscle cells express $P2X_1$, $P2X_2$, $P2X_4$, $P2X_7$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ (Kunapuli & Daniel, 1998; Cario-Toumaniantz *et al*., 1998). It is uncertain whether they also express the $P2Y_1R$ (Kunapuli & Daniel, 1998; Erlinge, 1998), and there are no reports on $P2Y_{11}$ expression. While great emphasis is given to P2R present on the endothelium and the smooth muscle cell layer, expression of P2R by fibroblasts, another key cellular component of the vessel wall, should not be overlooked, especially in pathologic conditions. Human and rat fibroblasts are known to express $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2X_3$, $P2X_4$ and $P2X_7$, likely in an activation-dependent fashion (Webb *et al*., 1996; Zheng *et al*., 1998; Solini *et al*., 1999; Solini & Morelli unpublished data). It is obvious that the final outcome of vascular stimulation by nucleotides depends on the integration of all the responses

Table 1

Mammalian P2Y and P2X receptors

Subtype	*Amino acid number*	*Preferred naturally occurring agonist*	*Signal transduction*
$P2Y_1$	362	ADP	IP_3, cAMP
$P2Y_2$	373	UTP, ATP	IP_3,
$P2Y_4$	352	UTP	IP_3
$P2Y_6$	379	UDP	IP_3
$P2Y_{11}$	371	ATP	IP_3, cAMP
$P2Y_{12}$	342	ADP	cAMP
$P2Y_{13}$	334	ADP	IP_3, cAMP
$P2X_1$	399	ATP	Ion currents
$P2X_2$*	472	ATP	Ion currents
$P2X_3$	397	ATP	Ion currents
$P2X_4$*	388	ATP	Ion currents
$P2X_5$	455	ATP	Ion currents
$P2X_6$	379	ATP	Ion currents
$P2X_7$	595	ATP	Ion currents protein–protein interaction
$P2X_2/P2X_3$		ATP	Ion currents
$P2X_1/P2X_5$		ATP	Ion currents
$P2X_4/P2X_6$		ATP	Ion currents

*Splice variants of $P2X_2$ and $P2X_4$ have also been identified. Funtional and molecular evidence exists for the presence of heteromeric P2X receptors (see Surprenant & North, 2000). At $P2Y_1$ and $P2Y_{12}$, ATP may act as an antagonist. $P2Y_1$ and $P2Y_{12}$ mediate inhibition of adenylate cyclase; $P2Y_{11}$ mediates stimulation of adenylate cyclase; $P2Y_{13}$ mediates inhibition of adenylate cyclase at low and stimulation at high concentrations.

elicited by individual P2Y and P2X receptors expressed on all the cell elements of the vessel wall.

P2 receptors in blood cells

Blood cells express P2 receptors of both the P2Y and P2X subfamilies. In the P2YR subfamily, $P2Y_1$, $P2Y_2$, $P2Y_6$ and $P2Y_{11}$ were reported to be expressed by monocytes, B lymphocytes or polymorphonuclear granulocytes (Di Virgilio *et al*., 2001). Monocytes and macrophages also express $P2X_1$, $P2X_4$ and $P2X_7$, while in granulocytes only expression of the $P2X_7$ subtype has been reported (Suh *et al*., 2001). T lymphocytes express a $P2X_7$-like receptor, while it is still controversial whether normal B lymphocytes express functional P2XR (Baricordi *et al*., 1996; Gu *et al*., 2000), although a recent confocal microscopy study has revealed expression of the PX_1, $P2X_2$, $P2X_4$ and $P2X_7$ proteins (Sluyter *et al*., 2001). Dendritic cells generated *in vitro* from monocyte precursors express $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2X_1$, $P2X_4$, $P2X_5$ and $P2X_7$ (Liu *et al*., 1999; Berchtold *et al*., 1999; Ferrari *et al*., 2000b). Platelets express $P2Y_1$, $P2Y_{12}$ and $P2X_1$ (Sun *et al*., 1998; Leon *et al*., 1997; Hollopeter *et al*., 2001). Erythrocytes are known to express a $P2X_7$-like receptor and, at least in turkey, a $P2Y_1$-like receptor (Parker & Snow, 1972; Boyer *et al*., 1989).

Following migration and/or stimulation with inflammatory mediators, pattern of expression in leukocytes may change substantially, leading to downmodulation of some subtypes (e.g. $P2Y_1$) and upmodulation of others (e.g. $P2X_7$) (Dubyak *et al*., 1996; Humphreys & Dubyak, 1996). The only known physiological activator of P2XR is ATP; on the contrary, the six P2YR subtypes are differentially sensitive to various nucleotides: at $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ the preferred agonist is ADP, at $P2Y_2$ ATP and UTP are equipotent, at $P2Y_4$ UTP is preferred, at $P2Y_6$ UDP, while $P2Y_{11}$ is the only P2YR selective for ATP (von kugelgen & Wetter, 2000; Communi *et al*., 2001). A relevant consequence of such a nucleotide selectivity is that even on the very same cell, P2Y receptors are differentially activated depending on the extracellular nucleotide milieu to which the cell is exposed. For example, where extensive conversion of extracellular ATP to ADP takes place, as it occurs at sites of platelet aggregation, $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ should be the main receptors activated, while on the contrary at sites exposed to shear-stress forces, where ATP is the principal nucleotide released, $P2Y_{11}$ is likely to be the main receptor stimulated. It should also be pointed out that ATP may act as an antagonist at $P2Y_1$ and $P2Y_{12}$ (von kugelgen & Wetter, 2000). There is evidence that UTP is released *via* non-lytic pathways from a variety of cells (platelets, leukocytes, epithelia) (Lazarowski & Harden, 1999), and it has to be expected that cell membrane damage causes efflux into the pericellular milieu of both ATP and the other intracellular nucleotides, UTP and UDP included, thus it is possible that at sites of cell damage $P2Y_4$ and $P2Y_6$ are stimulated. An additional family of agonist molecules are the diadenosine polyphosphates, that are often co-released with ATP and ADP and stimulate P2 receptors, either directly of after breakdown to ATP (Rodriguez del Castillo *et al*., 1988; Miras-Portugal *et al*., 1999).

Another factor that modulates P2-mediated responses is the local nucleotide concentration: P2YR have usually high affinity for the ligand, and are therefore responsive to nanomolar concentrations (Ralevic & Burnstock, 1998), while on the contrary $P2X_{1-6}R$ have EC_{50} in the low micromolar range, and $P2X_7$ in the hundred micromolar range (Khakh *et al*., 2001). This may suggest that P2 receptors that mediate release of pro-inflammatory factors or trigger a cytotoxic effect, such as $P2X_7$, are not usually activated in conditions under which other members of the P2 family are fully active. This confers a great plasticity to a nucleotide-based extracellular signalling system.

An alternative, and as yet almost unexplored, pathway for increasing the extracellular ATP concentration is a transphosphorylation reaction whereby ATP is synthesized at the expenses of AMP or ADP. This mechanism has been reported to be active on the surface of cultured human umbilical vein endothelial cells, and might therefore contribute substantially to accumulation of extracellular ATP (and possibly other nucleotides) (Yegutkin *et al*., 2001).

Systems involved in the degradation of extracellular ATP

As it would be expected for a highly regulated signalling network, extracellular ATP is a substrate for powerful hydrolytic enzymes that shorten drastically its lifespan. A recent classification by Zimmermann identifies four families of enzymes involved in the hydrolysis of extracellular nucleotides: ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase), ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP), alkaline phosphatase and ecto-5′-nucleotidase (Zimmermann & Braun, 1999; Zimmermann, 2000).

E-NTPDase is a large family that can be subdivided into two subgroups according to the membrane topology: members of the first group are predicted to have two hydrophobic transmembrane domains, while members of the second group have only one transmembrane domain, with a large COOH residue facing the extracellular environment. E-NTPDases hydrolyze ATP, ADP, several other purine and pyrimidine nucleotides, and are also known as differentiation markers of lymphocytes (CD39). The E-NPP family includes three members (NPP1, NPP2 and NPP3) that are also known as plasma cell differentiation antigens, motility stimulating proteins (autotaxin) or neural differentiation and tumor surface markers (Zimmermann, 2000). These enzymes cleave 3′, 5′-cyclic AMP to AMP, ATP to AMP and PP_i, ADP to AMP and P_i or NAD^+ to AMP and nicotinamide mononucleotide. In addition, they also hydrolyze pyrimidin nucleotides as well as the phosphodiester bonds of nucleic acids and the pyrophosphate bond of nucleotide sugars. E-NPP have a single transmembrane domain with an extracellular COOH terminus. Alkaline phosphatases are a family of non-specific ecto-phosphomonoesterases with a broad substrate specificity. Besides degrading nucleoside 5′-tri, -di, and -monophosphates, they also degrade PP_i and a large number of phosphorylated substrates. Alkaline phosphatases are glycosylphosphatidylinositol (GPI)-anchored membrane protein. Ecto-5′-nucleotidase is also a GPI-anchored molecule known as a lymphocyte maturation marker (CD73). This enzyme catalyzes the conversion of nucleoside 5′-monophosphates to the respective nucleosides an P_i, and is the main enzyme responsible for the generation of adenosine.

Extracellular nucleotide-hydrolyzing enzymes do not show a selective tissue distribution and are often found colocalized

on the same cells. Soluble forms are also present. Functional role of enzymes involved in metabolism of extracellular ATP is not entirely understood, with the exception of a few examples where their activity appears crucial such as platelet aggregation (Enjyoji *et al*., 1999), inflammation (Kaczmarek *et al*., 1996; Robson *et al*., 1997), ischaemia (Braun *et al*., 1998), calcification (Okawa *et al*., 1998), cell motility (Murata *et al*., 1994) or adhesion (Airas *et al*., 1997). CD39 is a potent inhibitor of platelet aggregation since this enzyme converts pro-aggregatory ADP to anti-aggregatory adenosine (Kaczmarek *et al*., 1996; Marcus *et al*., 1997). Recombinant CD39 inhibits ADP and collagen-induced platelet aggregation, suggesting that soluble forms of this enzyme might be useful as anti-thrombotic drugs. Similarly, intravenous administration of apyrase might have anti-aggregant effects of potential therapeutic value.

P2 receptor-dependent release of inflammatory mediators from blood cells

Stimulation of P2 receptors is coupled to release of a wealth of inflammatory mediators (see Di Virgilio *et al*., 2001, for a recent review). Of obvious relevance for atherosclerotic plaque formation and progression, is release of the pro-inflammatory cytokines IL-1β, IL-1α, IL-6, IL-8, and TNFα (Perregaux & Gabel, 1994; 1998; Ferrari *et al*., 1997a; Solini *et al*., 1999; Ferrari *et al*., 2000b; Warny *et al*., 2001). Furthermore, nucleotide receptors may also mediate up-regulation of NO synthase and NO generation (Tonetti *et al*., 1994; Denlinger *et al*., 1996; Hu *et al*., 1998). In neutrophilic and eosinophilic polymorphonuclear granulocytes, P2 receptor stimulation triggers superoxide anion generation and exocytosis of both specific and azurophilic granules (Cockcroft & Stutchfield, 1989; Balazovich & Boxer, 1990; Ferrari *et al*., 2000a; Suh *et al*., 2001). Nucleotide stimulation also increases membrane expression of CD11b/CD18 and cell adhesion to albumin-coated latex beads (Freyer *et al*., 1988). Presence of the integrin-binding domain RGD in the first extracellular loop of $P2Y_2$ (Erb *et al*., 2001) strengthens involvement of P2 receptors in cell adhesion. Platelets are a key target for nucleotides in the blood (Hourani & Hall, 1994). Three P2 receptors expressed on platelets ($P2X_1$, $P2Y_1$ and $P2Y_{12}$) have a major role in the regulation of platelet aggregation and thrombus formation (Kunapuli, 2000; Savi & Herbert, 2000; Hollopeter *et al*., 2001). ADP, the main agonist nucleotide for platelets, causes shape changes, aggregation, thromboxane A_2 formation and granule secretion.

On endothelial cells extracellular nucleotides have multiple effects, such as modulation of proliferation and angiogenesis (van Daele *et al*., 1992; Rathbone *et al*., 1992), PGI_2 and NO generation (Boeynaems & Galand, 1983; Mathie *et al*., 1991; Yagi *et al*., 1994), release of von Willebrand factor (Vischer & Wollheim, 1998) and tissue-type plasminogen activator (Hrafknelsdottir *et al*., 2001). Besides their action on endothelium, nucleotides also have a well documented action on two key components of the vessel wall: SMCs and fibroblasts (Wang *et al*., 1991; Erlinge, 1998; Harper *et al*., 1998; Solini *et al*., 1999; 2000 White *et al*., 2000). In smooth muscle cells, nucleotides act as mitogens or co-mitogens in synergism with polypeptide growth factors or neuropeptides. Of particular relevance is synergism with PDGF, a factor with a well defined role in atherogenesis. Furthermore, the potent pro-inflammatory cytokine IL-1β up-regulates the $P2Y_2$ receptor subtype and increases the mitogenic response to UTP (Hou *et al*., 2000). This is of obvious relevance in view of the inflammatory etiology of the atherosclerotic plaque. Little attention has been paid to fibroblasts both as a possible target of extracellular nucleotides in the vessel wall, and as a possible source of inflammatory mediators. Fibroblasts are known to produce several cytokines and pro-inflammatory factors, besides being very active in the deposition of extracellular matrix. We have recently shown that human primary fibroblast cultures release substantial amount of IL-6 upon stimulation with extracellular ATP, likely *via* activation of $P2X_7$, and this activity is potentiated in conditions mimicking hyperglycaemia (Solini *et al*., 1999; 2000). However, since human fibroblasts express several other P2 receptors, besides $P2X_7$ ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2X_3$ and $P2X_4$), a wider range of responses to nucleotide stimulation is anticipated. It would be of interest to investigate if and to what extent P2 receptor stimulation could also cause secretion of extracellular matrix constituents such as laminin, collagen or elastin. An additional aspect of interest would be the ability of P2 receptor agonist to stimulate fibroblast proliferation and/or differentiation into smooth muscle cells.

A hypothesis for a role for P2 receptors in atherosclerosis

Activation of endothelial cells and their interaction with blood cells is one of the key factors in the pathogenesis of atherosclerosis. Ample evidence supports the interpretation that local inflammatory changes that involve the endothelium, circulating cells as well as elements of the arterial wall have a crucial role in plaque progression and instability. It is increasingly appreciated that the luminal side of the endothelium is exposed to variable ATP levels, that are only grossly reflected by the ATP concentration, up to 20 μM, measured in bulk plasma (Born & Kratzer, 1984). The endothelial cells themselves are capable of ATP release under the effect of shear stress forces (Bodin *et al*., 1991), swelling (Oike *et al*., 2000), or stimulation of plasma membrane receptors (Yang *et al*., 1994) (Figure 3). In addition, since ATP is stored within platelet dense granules, massive amounts of ATP are released during platelet aggregation on the endothelial surface (Meyers *et al*., 1982; Lages & Weiss, 1999). The level of extracellular ATP measured at sites of massive platelet aggregation (20–50 μM) is clearly an underestimation of the concentration that could be reached if degranulation occurred in a protected compartment between the platelet and endothelial cell plasma membranes. Besides endothelial cells and platelets, leukocytes are an additional important source of extracellular ATP. Although it is still a debated issue (see for example Beigi & Dubyak, 2000), substantial evidence is accumulating to support the ability of inflammatory mediators (e.g. bacterial endotoxin, LPS) to cause ATP release from leukocytes and endothelial cells (Ferrari *et al*., 1997b; Sperlagh *et al*., 1998; Sikora *et al*., 1999; Imai *et al*., 2000; Warny *et al*., 2001). This suggests that purinergic stimulation can also occur in the vessel wall in response to transient or long lasting bacteraemia, or to the deposition of septic emboli. A purinergic loop could also be triggered by mechanical insults, especially if associated to the concomitant administration of high cholesterol diet, as

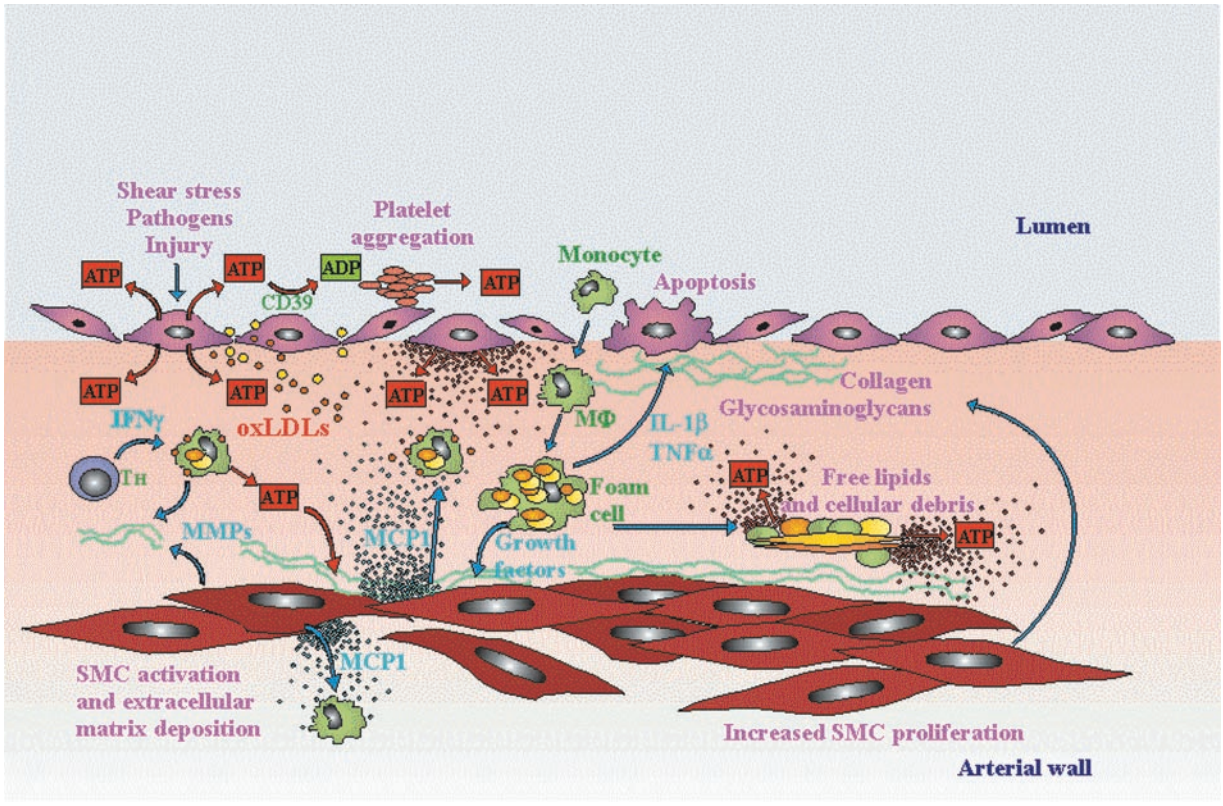

Figure 3 Hypothetical role of extracellular ATP and nucleotide receptors in atherosclerotic plaque formation. Endothelial damage or activation causes ATP release both into the blood and the arterial wall. On the endothelial surface ATP is hydrolized to ADP thus triggering platelet aggregation *via* $P2Y_1R$ and $P2Y_{12}R$. Activated platelets release further ATP that feed-backs on the endothelium increasing its state of activation. In the subintimal space ATP synergyzes with other agents (e.g. oxLDL or IFNγ released by T helper lymphocytes) to promote macrophage activation. ATP acting at P2YR also contributes with MCP-1 to the formation of a chemoatctic gradient for monocytes and macrophages. Macrophages are recruited from circulation as well as from the arterial wall. Under stimulation with ATP, SMCs proliferate and release MMPs and extracellular matrix proteins. ATP, acting at $P2X_7R$ and presumably also at P2YR triggers IL-1β and TNFα release from activated macrophages. At the same time, a high level of extracellular nucleotide released by activated or damaged cells causes a sustained activation of the $P2X_7R$, thus accelerating cell death, either by necrosis or apoptosis.

suggested by experiments showing a dramatic redistribution of P2XR in rabbit aorta subjected to balloon injury (Pulvirenti *et al*., 2000). Finally, human primary fibroblast cultures *in vitro* spontaneously release ATP (A. Solini *et al*., submitted), therefore it can be assumed that ATP is also secreted by fibroblasts within the arterial wall.

It is evident that presence of ATP in the pericellular space must be considered the rule rather than the exception, although a rigorous quantitative determination of the actual concentration of this nucleotide has never been performed. *In vitro* measurements of ATP released from different cell types indicate a bulk solution concentration in the nanomolarlow micromolar range, however the local concentration of ATP in the vicinity of the plasma membrane might be substantially higher. Beigi *et al*. (1999) measured the local ATP concentration with a luciferase tethered to the platelet plasma membrane, obtaining a peak release, after stimulation with thrombin, in the 15–20 μM range. This concentration is sufficient to trigger on all P2Y and P2X receptor subtypes, with the exception of $P2X_7$. However, it has been reported that $P2X_7R$ undergoes a progressive increase in current (current growth) in response to repeated stimulation with submaximal agonist (ATP or benzoyl ATP) doses, presumably due to either increase in pore size or in agonist potency (or both) (Surprenant *et al*., 1996; Rassendren *et al*., 1997; Chessell *et al*., 1997; Hibell *et al*., 2000). This observation suggests that *in vivo* even low but repeated pulses of ATP might be able to activate $P2X_7R$. The requirement for a high ATP dose or repeated ATP pulses would be in keeping with the hypothesis that the $P2X_7R$ should be quiescent under normal physiological conditions and only be activated when extensive tissue damage or activation of the inflammatory system occur (Di Virgilio, 1995; Di Virgilio *et al*., 2001). Indirect evidence that ATP release in circulation occurs at a level at least sufficient to activate P2YR is provided by the surprising phenotype of the *cd39*/ATP diphosphohydrolase knockout ($cd39^{-/-}$) mouse. In these animals the ability to degrade extracellular ATP and ADP is severely impaired, thus it was expected that they should exhibit a thrombotic diathesis due to pronounced platelet stimulation by ADP. On the contrary, the $cd39^{-/-}$ mouse shows a prolonged bleeding time and failure to undergo platelet aggregation. These defects are due to $P2Y_1R$ desensitization dependent on the increased accumulation of extracellular ATP, and are largely corrected by the administration of apyrase (Enjyoji *et al*., 1999). In addition, factors predisposing to atherosclerosis might contribute to prolong the half life of extracellular ATP in the vasculature, since it has been reported that activity of

endothelial ATP diphophohydrolase is lost when endothelial cells are exposed to inflammatory mediators that cause generation of oxygen radicals (Robson *et al*., 1997).

Under normal physiological conditions, local ATP release from the endothelium in response to shear stress forces or locally released neuromediators has an important role in the regulation of blood flow. It is well documented that ATP is a potent NO and PGI_2 releasing agent, and thus a vasodilatatory stimulus (Burnstock, 1999; Burnstock & Williams, 2000). However, prolonged stimulation of endothelial cells by haemodinamic stress, or platelet aggregation on the endothelial surface, may cause a large and sustained ATP release that may initiate an 'inflammatory' activation of the endothelium. Nucleotides have been reported to cause upregulation of CD11b/CD18 on granulocytes and enhance adherence of leukocytes to latex beads and endothelial cells (Freyer *et al*., 1988; Oryu *et al*., 1996). Enhancement of the adhesive properties of the endothelium favours platelet aggregation and the release of platelet-derived pro-atherosclerotic factors. Release of ATP within the vessel wall may also participate in the generation of an inflammatory microenvironment. Besides endothelial cells, sources of ATP may be vascular smooth muscle cells or fibroblasts. Furthermore, as discussed above, inflammatory cells migrating into the nascent atherosclerotic plaque are another potentially important source of extracellular ATP. Accumulation of this nucleotide at this stage might contribute to plaque formation *via* several potential mechanisms: (a) by generating a chemotactic gradient for migration of monocytes into the nascent plaque; (b) by synergyzing with other growth factors to stimulate and sustain SMC growth; (c) by activating, alone or in concert with other stimuli released into the vessel wall, effector functions of inflammatory cells migrated into the plaque.

Nucleotides participates to the formation of a chemotactic gradient both directly (mononuclear phagocytes are known to be attracted towards sites of ATP or UTP release) (Oshimi *et al*., 1999), and indirectly, by stimulating the release of chemokines such as IL-8 (Warny *et al*., 2001). A local environment characterized by a high extracellular nucleotide concentration favours proliferation of SMCs and macrophages, and may thus contribute to the expansion of the cellular component of the plaque. ATP-dependent release of cytokines such as IL-1β and TNFα from macrophages may have a major role in the induction of early alterations and in the progression of atheromatosis. In addition, ATP also triggers release of NO and oxygen radicals from macrophages, agents that may have a cytotoxic effect on the arterial wall.

Extracellular ATP itself might have a relevant cytotoxic role within the plaque. It is well known that a crucial step in atheroma progression is lysis of lipid-laden macrophages (foam cells) with the ensuing release of their content into the necrotic core. Many different precipitating factors are involved in this process, one of which could be the sustained stimulation of the $P2X_7R$, a molecule expressed to a high level in macrophages and well known for its potent cytotoxic activity (Murgia *et al*., 1992; Di Virgilio *et al*., 1998). Stimulation with inflammatory cytokines (e.g. IFNγ) enhances expression of some P2R subtypes (e.g. $P2X_7$), and thus is expected to increase cell susceptibility to the cytotoxic effect of ATP (Falzoni *et al*., 1995). In the cell infiltrate of the atherosclerotic plaque the presence of T_H lymphocytes is well documented. These cells are a main source of IFNγ during inflammation and therefore they may affect P2 receptor expression by the macrophages. This may trigger an amplification loop that exacerbates tissue damage. Particular disease conditions may further enhance the contribution of extracellular ATP to the necrotic degeneration of the plaque. Recent experiments have shown that fibroblasts cultured in *in vitro* conditions that mimick hyperglycaemia are exquisitely sensitive to the cytotoxic effect of ATP (Solini *et al*., 2000). Furthermore, ATP causes a large release of IL-6 from this cells primed with LPS and phorbol esters. IL-6 is another cytokine that is believed to have a central role in the progression of the plaque and in the acceleration of the arterial lesions observed during diabetes (Morohoshi *et al*., 1996). Therefore, it is possible that a shift in nucleotide sensitivity is a contributing factor in the pathogenesis of atherosclerotic lesions in diabetes and in other metabolic diseases.

The P2R and extracellular nucleotides might also have a distinct role in the transition from a latent to an overt atherosclerotic disease. It is in fact known that while atherosclerotic lesions are fairly wide spread throughout the population, only a few subjects develop a full blown ischaemic disease. This is thought to be due to the destabilization of the plaque, a phenomenon not necessarily involving rupture. Destabilization causes an alteration in the properties of the endothelial lining that favours thrombosis. Extracellular nucleotides might contribute to this phenomenon in a dual capacity: on the one hand by inducing an increased release of IL-1β and TNFα from inflammatory cells (it is not known whether the endothelium itself might be a source of IL-1β), and on the other by generating a local proaggregant environment at the endothelial surface, or even by causing endothelial cell apoptosis (von Albertini *et al*., 1998; Goepfert *et al*., 2000; Mallat & Tedgui, 2000). In the complex inflammatory status of the atherosclerotic plaque, MMPs are enjoying increasing interest for their role in the digestion of extracellular matrix and in the weakening of the fibrous cap. These proteases are secreted into the plaque by macrophages, SMCs and lymphocytes, but factors promoting their release and activation are poorly known. Extracellular ATP, acting at the $P2X_7R$, causes a large activation of a MMP expressed on the surface of B lymphocytes (Gu *et al*., 1998a), therefore it is not unlikely that this nucleotide might also participate in the stimulation of protease activity within the plaque.

Conclusions

Nucleotide receptors are emerging as increasingly important molecules in regulation of the physiology of platelets, leukocytes and cells of the vessel wall. Large clinical trials performed with antagonists of the platelet ADP receptor (ticlodipine and clopidogrel) in patients with atherosclerotic diseases have shown a significant benefit compared to aspirin (Balsano *et al*., 1990; Jarvis & Simpson, 2000; Savi & Herbert, 2000). New selective and potent antagonists at the various P2 subtypes are actively investigated and have entered experimentation as well as clinical trials, especially as antithrombotic drugs (e.g. ARL 67156, AR-C69931MX and R-99224) (Burnstock & Williams, 2000; Sneddon *et al*., 2000; Storey, 2001; Sugidachi *et al*., 2001). The identification of the P2 subtypes expressed by leukocytes, SMCs, endothelial cells and fibroblasts may allow selective inhibition

of certain unwanted responses (e.g. cytokine release or proliferation) without affecting others (e.g. release of vasodilatory agents). Furthermore, it is now clear that ectoATP/ADPases and ecto nucleotidases are another promising target for the modulation of platelet aggregation and thrombus formation (Marcus *et al*., 2000). Thus, from the integration of current awareness on the inflammatory aetiology and pathogenesis of the atheromatous plaque and the increasing knowledge of the complex network of the extracellular nucleotides we can expect the design of innovative approaches to the prevention and treatment of atherosclerosis.

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