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The cell and molecular biological approach to biomaterial research: a perspective

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The past two decades have witnessed a revolution in our understanding of chemical processes in living organisms. This is mainly a result of the massive advances in the fields of cell and molecular biology. These techniques are highly relevant to the biomaterials sector, as they offer the scientist the possibility to better understand the mechanisms involved in the interactions between cells and a material surface—a prerequisite for the rational development of medical devices with optimal biocompatibility. The purpose of the present article is to explain the rationale of the cell and molecular biological approach to biomaterial research and to present typical examples from the authors' laboratory, as well as from the literature, to illustrate its application. Important aspects of interfacial biology, including the underlying biological mechanisms and methodology, are presented. Of the latter the combination of morphological techniques with methods of cell and molecular biology as well as molecular genetics (so-called "combinative techniques") are particularly useful. The applicability of this approach is illustrated from a study on the pathomechanisms of metal ion-induced inflammation. In addition, the approach is essential to the development of targeted intervention strategies, as for example in the luminal surface modification of vascular prostheses to permit endothelial cell seeding.

1. Introduction

> The strength of all sciences, which consists in their harmony, each supporting the other....
>
> Francis Bacon (1561–1626)

The interdisciplinary nature of biomaterial research is probably the greatest asset which this field of intellectual endeavour possesses, but is at the same time a seemingly insurmountable barrier to progress, on account of the breadth of its scientific base. The latter encompasses many of the non-biological sciences as well as the entire field of biomedicine, with the result that an engineer has difficulty understanding the language of a vascular surgeon and vice versa. The past twenty years have witnessed a revolution in biomedical research, with the advent and rapid expansion of cell and molecular biology. This has transformed the entire approach to studying the development of diseases (pathogenesis) and has led to deepening understanding of processes as diverse as oncogenesis and atherosclerosis. The present authors hold the firm view that the future of biomaterial research for human application will be markedly influenced by this approach and that up until now too few of the major advances in cell and molecular biology have been applied to the biomaterials field.

The purpose of the present article is twofold, namely to define the scope of the cell and molecular biological approach and to present evidence for its relevance to the advancement of medical device development. In doing so, research from our laboratory will be presented to underline the significance of this approach and will concern the induction of inflammation by implanted metals, as well as the endeavours to improve endothelial cell seeding of vascular prostheses. Although this paper will have of necessity review character, it will present data which have not as yet been published. In the interest of clarity, the scientific methodology adopted will be presented in extended figure legends.

2. Interfacial biology: the common denominator following implantation

Irrespective of whether the biomaterial employed is designed to permit drug delivery, is meant to be biodegraded or act as a permanent implant, the common factor following implantation is the reaction which

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occurs at the interface between the implant and the host organism. The study of the biological reactions which subsequently take place can be termed *interfacial biology*. Interfacial biology may be viewed as having two principal foci of interest, which for the sake of clarity may be formulated as fundamental questions:

1. What is the *biomaterial* doing to the organism?
A good example of this is the biomaterial-induced thrombosis following implantation of blood-contacting devices (Fig. 1) or use of an extracorporeal circulation [1, 2].
2. What is the *organism* doing to the biomaterial?
This alternative viewpoint is well highlighted by the phenomenon of enzymatic degradation and is highly relevant to biomaterial research, as phagocytes, such as macrophages and neutrophilic polymorphonuclear granulocytes (PMN), form part of the unspecific immune response to a foreign body [3, 4]. These cells are equipped with a battery of efficient enzymes and oxygen radicals with the capacity to attack substances as diverse as metals and synthetic polymers [5–8].

To understand the events occurring at the interface between a biomaterial and the living organism, it is necessary to dissect the possible interactions into a series of mechanisms, involved in the induction of cell activation.

Figure 1 Mitral valve prosthesis *in situ*, viewed from the lumen of the left ventricle, in a 73-year old female. The ventricular aspect of the valve is to a large extent covered by thrombotic material. This deposition led to acute cardiac failure and death of the patient. The upper left of the figure shows the three intact cusps of the aortic valve.

Biomolecular adsorption and desorption on the biomaterial surface

The initial events concern the interactions between biomolecules and the biomaterial surface and involve, on the whole, blood proteins/glycoproteins, which sequentially adsorb and desorb on the material surface. Vroman [9] demonstrated very clearly that this process is highly dynamic. There is also good evidence that adhesive proteins also compete with each other in the adsorption process [10]. There can be no doubt that these events are the prime determining factor in controlling the subsequent cellular interactions and is probably the most neglected field of interfacial biology to date, although recently new methods are being applied to study protein adsorption, such as modified immunoblotting [11].

It should be stressed that the vast majority of these studies have been carried out *in vitro*. While not definitively proven, it is assumed that similar processes are decisive for cell-biomaterial interactions *in vivo*. Nevertheless, the complex *in vivo* situation presents problems, such as the differences in implant site. Thus, although with all forms of implantation, blood exposure is inevitable, it is evident that blood-contacting medical devices and implants in solid tissues will encounter a different spectrum of body proteins and therefore presumably evoke different cellular responses.

Cell adhesion mechanisms

The adsorbed proteins on the biomaterial surface determine how various cell types become adherent to the surface [12–15]—a pre-requisite for subsequent cell activation. The process of adhesion involves the recognition of the biomolecules by specific receptors on the cell surface. These receptor molecules are usually members of the integrin family, heterodimeric membrane glycoproteins, composed of two subunits, alpha and beta [16, 17].

Cell activation mechanisms

Following cell adhesion there are various possibilities for cell activation, including spreading, migration, proliferation and the induction of cellular biosynthetic activity. Which of these activities occur depends not only on cell type, but also on the composition and molecular conformation of the biomolecular coating, which is controlled by the physical and chemical properties of the material surface [18–20].

Cell recruitment

This phenomenon is a result of a particular type of cell activation, but deserves special attention, as it is an amplification mechanism which can have negative consequences for the success of the biomaterial implant. Two aspects are worthy of interest:

Chemotactic factor expression Certain cells produce so-called chemotactic agents, which set up a chemical gradient detectable by cells of similar or different type, leading to movement along this gradient [21]. Thus, for example, biomaterial-induced activation of a neutrophilic granulocyte can lead to release of leukotriene B4, which can chemotactically attract other neutrophils [22]. Although this is a part of the physiological response to injury, the process could be sustained by continuous granulocyte activation by the biomaterial surface.

Expression of cell adhesion molecules (CAMs) The discovery of a variety of families of molecules on the

surface of cells which regulate cell–cell interactions has transformed our view of how cells communicate with each other [23, 24]. This phenomenon is very relevant to our understanding of how biomaterials evoke particular patterns of cellular reaction following implantation and may enable therapeutic strategies to be developed to inhibit undesirable cellular reactions. This theme is of such importance that it will be addressed more fully in the context of how biomaterials can induce inflammation (see below).

2.1. Interfacial biology: methodology

There are three principal methods available for the study of interfacial biology:

Explantation in humans

While being the most relevant of all three methods, it is dogged by the problem that material only becomes available on a sporadic basis, so that it is practically impossible for one centre to carry out a statistically significant study within an acceptable time-frame. The only feasable alternative is to establish well-coordinated retrieval centres, the value of which cannot be over-emphasized.

Animal experimentation

This remains a mainstay of the investigation of how biomaterials interact with the living organism, although it is hoped that the number of such experiments may be restricted by expanding point 3 (below). Nevertheless, it is evident that implantation in the intact animal is vital for complex interactions, such as load-bearing in bones and joints [25].

In vitro techniques

The application of tissue culture techniques to the study of interfacial biology is gaining in importance and represents an invaluable source of information on cell behaviour on biomaterials. It is nevertheless fraught with the problem of relevance to the human situation [26], as well as the lack of concerted effort to develop the methodology from static culture systems to more complex dynamic situations [27].

For both human explanation and animal experimentation great attention must be paid to the preservation of the true interface between host tissue and the implant itself. This presents the research worker with considerable practical problems, including how to deal with differences in compliance of tissue and material [28], as well as how to remove materials which cannot be sectioned, such as metals [29]. Various ingenious solutions have been found to preserve metal–bone interfaces and include the ground section/sawing technique [30] and the electropolishing method [31]. Practical problems are also encountered *in vitro*, if TEM studies are to be performed on cells in contact with metals [32].

The study of interfacial biology must be aimed at addressing the following questions:
which cells are present?
In which temporal sequence do they appear? and
What are the various cells doing? The latter is not as trivial as it might appear at first sight. It is essential to distinguish between *active participants* in the biological processes occurring at the interface and *passive bystanders*, which theoretically could numerically be in the majority and thus mask the primary cells of interest.

2.2. Interfacial biology: available techniques

The techniques applicable to the study of interfacial biology are numerous, but for the sake of clarity they can be subdivided into four principal groups.

Morphological techniques

Conventional morphology ranks as one of the most useful methods to study how implanted biomaterials interact with the host organism. This study can be conducted at both light microscopical and ultrastructural level. The latter includes conventional scanning (SEM) and transmission electron microscopy (TEM), as well as more sophisticated techniques such as intermediate voltage EM (IVEM) and X-ray microanalytical methods to detect certain elements. In addition, there is the possibility of combining the basic technique with image analysis to enable quantitation. The latter is an essential component of comparative studies of host interactions with biomaterials. Fig. 2 illustrates how conventional SEM can be employed to study the host reaction to an implant, in this case to a breast implant.

Biochemical analysis

Standard analytical techniques in biochemistry allow *gene products* to be quantitated. Many of the earlier methods of radioimmunoassay and various forms of chromatography have in recent years been augmented by the techniques of enzyme-linked immunosorbent assay (ELISA) and enzyme immunoassay (EIA). The latter methods are particularly useful for analysing gene products in tissue culture supernatants or in biological fluids

Molecular biology/molecular genetics

The fields of molecular biology (MB) and molecular genetics (MG) permit the researcher to study mechanisms of gene regulation. This involves specialized techniques to investigate nucleic acids (DNA, RNA), whether at macromolecular level or, in the case of DNA, as an integrated element of a chromosome. Fig. 3 presents a simplified scheme to illustrate how biochemical analysis and molecular biology are related to one another.

"Combinative" techniques

In our opinion this group of techniques represents one of the most powerful tools to study interfacial biology. The nomenclature is based on the fact that each

Figure 2 SEM photomicrograph of the "capsule" tissue around a polyurethane (PUR)-coated silicone breast prosthesis. This method demonstrates clearly that the PUR coating has been separated from the prosthesis. The figure shows sub-millimetre "spicules" of PUR embedded in a fibrous connective tissue matrix.

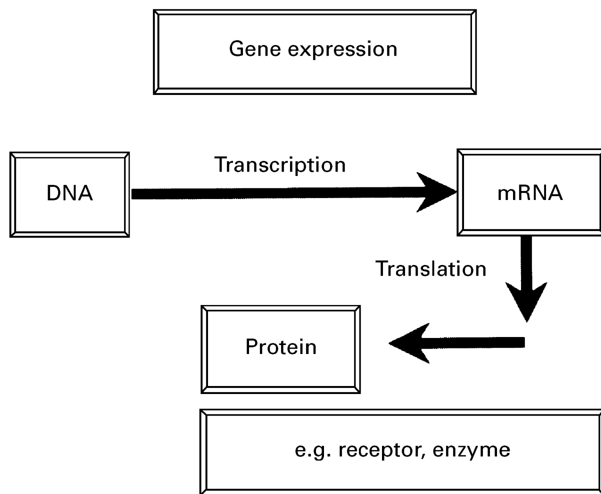

Figure 3 Simplified scheme of gene expression, in which an encoded gene (as a DNA sequence) is transcribed into a complementary messenger ribonucleic acid (mRNA), which is then translated into a gene product in protein form.

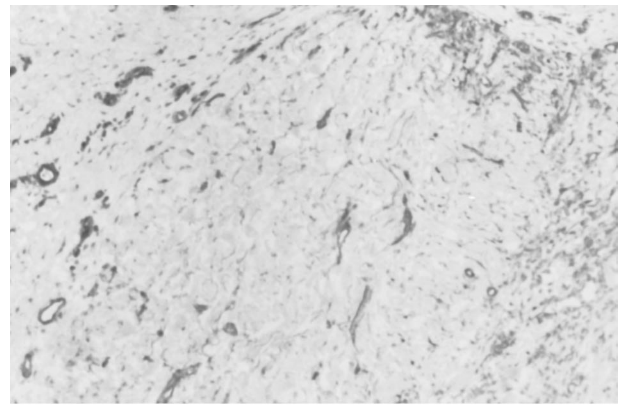

Figure 4 IH localization of blood vessel ingrowth into a collagen-elastin dermal substitute used to cover skin defects in a rat model. The tissue was harvested 11 days after biomaterial application *in vivo*. The tissue was cryosectioned at 5 μm and reacted with the Mab, MAS 259, which recognizes a rat endothelial cell epitope. The detection system employed an alkaline phosphatase reaction and gave a dark reaction product. ($\times$ 57)

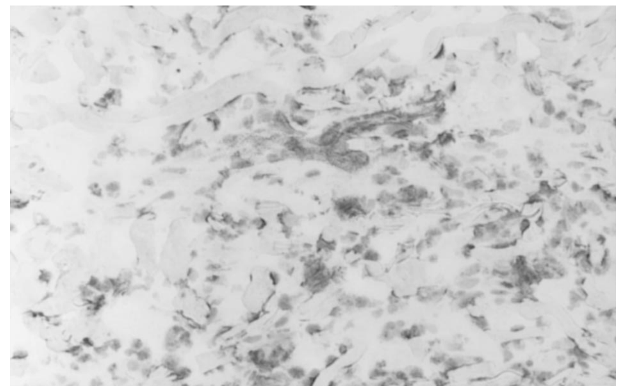

Figure 5 Experimental set-up *in vivo* in the rat as described for Fig. 4. Tissue was harvested 15 days post-operatively and macrophages localized in cryosections using the Mab, MCA 341, which recognizes a rat macrophage epitope. The alkaline phosphatase detection system gives a reaction product where the macrophage epitope is present. The photomicrograph was taken under partially polarized light to highlight the close association of the rat macrophages with the elastin component of the biomaterial (this appears as a faintly birefringent wavy material). ($\times$ 114)

method is a combination of more than one technique—a type of hybrid, in each case with the common factor morphology.

Morphology combined with analysis This combinative technique aims at localizing gene products and thus focuses on the translational level shown in Fig. 3. The advent of monoclonal antibody (Mab) technology has facilitated this development, which is termed immunohistochemistry (IH), if the localization is performed in a tissue section, or immunocytochemistry (IC), if performed on isolated cells. Localization at ultrastructural level is termed immunoelectron microscopy (IEM).

Morphology combined with MB/MG This method aims at studying the level of gene transcription and focusses on nucleic acids (DNA/RNA). Labelled "probes" containing the *complementary sequence* to the portion of a nucleic acid to be localized can be employed in a tissue or cell preparation (following unmasking of the nucleic acid to be localized) using the technique of *in situ* hybridization (ISH).

The value of these combinative techniques is that they are *in situ* methods, that is, they enable the *functional status* of a tissue or cell to be manifested while preserving the *structural integrity* of the cells. Figs. 4 and 5 illustrate how IH can be applied to study the reaction in vivo to a dermis substitute, consisting of both collagen and elastic elements. In Fig. 4 IH was used to study the degree of vascularization of the biomaterial, one of the essential reactions in the healing process and an element on the way to the formation of fibrous scar tissue [33]. Fig. 5 illustrates the IH localization of macrophage ingrowth into the biomaterial and clearly demonstrates the close association of macrophages with the elastic component of the biomaterial. Further ultrastructural studies

indicated that these macrophages phagocytose the elastic tissue and thus destroy one of the essential components of a dermal substitute. ISH will be illustrated under the heading of CAM expression (see below).

Despite the immense advantages offered by such combinative techniques, there are nevertheless certain drawbacks which should be mentioned. Thus, with the use of ISH it is possible to prove that, for example, a particular mRNA for a particular protein is present in a certain cell. Further essential questions arise, such as the identity of the cell concerned, as well as the necessity to state whether this mRNA is being translated into the final gene product. The mere presence of mRNA *does not prove* that the gene product is being synthesized. The solution of such problems are the (even) more complex combinative techniques of double and triple labelling, in which two or three reactions respectively in a cell can be demonstrated by using, for example, two or three different coloured reaction end-products. With these techniques it is possible to use one colour reaction for the ISH, and in a subsequent *in situ* reaction to use IH (with a second colour) with a cell marker antibody to detect the cell type involved. This could be, for example, a smooth muscle cell (use of smooth muscle actin as specific marker) or an endothelial cell (use of Ulex europeaus I lectin). It is then possible in the triple detection method to use a specific antibody directed against the gene product of the mRNA. This might be a cell adhesion molecule (CAM) on the cellular plasma membrane. In conclusion, it must be emphasized that such double and triple labelling techniques are among the most sophisticated methods used by morphologists and require very stringent methodological controls.

3. The cell and molecular biological approach: What use is it?

Having presented some of the important cell and molecular biological techniques applicable to interfacial biology, it is important to address the issue of the usefulness of this approach in biomaterial research. This can be seen as twofold:

1. the study of pathomechanisms, and related to this,
2. the development of strategies for "targeted intervention".

3.1. The study of pathomechanisms

The application of medical devices to the prevention and treatment of human disease is complicated by the fact that the body mounts a reaction to the biomaterial used. The mechanisms triggered in the living organism are often the same as those involved in many disease processes—the individual steps involved are referred to as "pathomechanisms" and their understanding is a task which falls in the realm of cell and molecular biology. This is nowhere better illustrated than in the complex issue of mutagenesis and carcinogenesis of biomaterials. Numerous studies on spontaneously arising human malignancies, as well as on induced tumours in animal models indicate that carcinogenesis is a multistep process, emanating from a problem of *genetic instability* [34–37]. This genetic instability manifests itself in a variety of ways, including gene deletion or inactivation of tumour suppressor genes [38] and activation, amplification etc. of oncogenes [39, 40]. These processes often involve mutation of genes [41]. All of these complex events can only be adequately understood by applying the expertise of cell and molecular biology.

3.2. Metal ion induction of inflammation

The significance of the aforementioned technologies for biomaterial research can be illustrated by a topic of considerable interest to the present authors, namely how implanted metals promote the inflammatory process. This problem is well known to maxillofacial, orthopaedic and trauma surgeons and is a complication which results in increased morbidity and therefore increased cost for the health services. In studying the metal-induced pathogenesis of inflammation, two approaches have been to investigate target cell (e.g. connective tissue cells) toxicity and alteration in cellular biosynthetic activity [42] as well as to study how metal ions affect the activity of inflammatory cells [43, 44].

The inflammatory process centres on the microcirculation, in which the endothelium plays a central role. The past decade has witnessed the rapid advancement of our understanding of the regulation of inflammation. A key role is played by the expression of a series of cell adhesion molecules (CAMs) on the surface of the endothelial cells (EC). Fig. 6 presents in schematic form four important CAM families. The *integrins*, especially those with the β1 subunit, are intimately involved in EC adhesion to the basement membrane [16, 17], while the *cadherin family* [45] in cooperation with other CAMs, for example PECAM-1, controls EC–EC interaction, which is vital for the integrity of the EC monolayer [46]. On the luminal, i.e. blood-contacting surface of the endothelium are two major CAM groups—the *immunoglobulin supergene family*, represented by the intercellular adhesion molecule-1 (ICAM-1) [47, 48] and the *selectins*, represented by endothelial-leukocyte adhesion molecule-1 (E-selectin) [23, 24, 49, 50].

CAMs such as ICAM-1 are present on normal EC, i.e. are *constitutively* expressed (Fig. 7). Such basal expression serves to regulate the physiological interaction between blood leukocytes and the blood vessel wall. During inflammation, ICAM-1 can be up-regulated to facilitate EC-leukocyte adhesion, which precedes leukocyte emigration out of the microcirculation towards an inflammatory focus. Molecules such as E-selectin are not expressed under physiological conditions. That means that they require *induction*, a process involving *de novo* mRNA synthesis, resulting in ultimate expression of the gene product, which then appears on the EC plasma membrane [51, 52]. This CAM can be localized using IEM on cultured EC under control (unstimulated) conditions, in which the

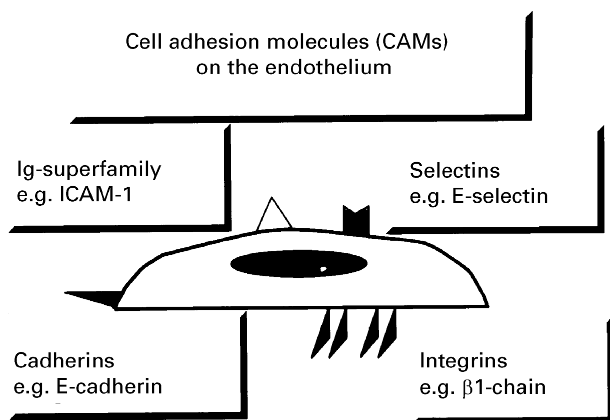

Figure 6 Schematic presentation of four major groups of CAMs expressed on the endothelium.

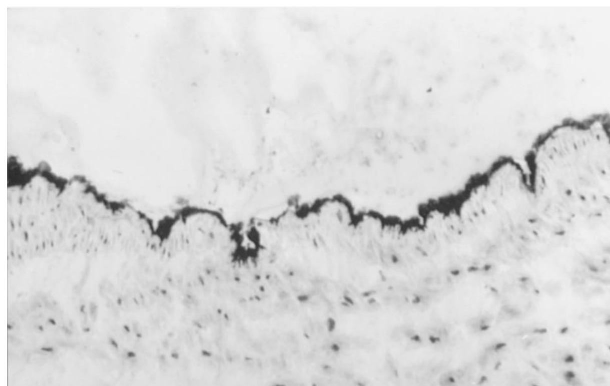

Figure 7 Immunohistochemical localization of one of the constitutively expressed CAM of the Ig supergene family, PECAM-1 (CD 31) (=platelet-endothelial CAM-1). The dark reaction product heavily marks the EC of a normal human umbilical vein. Cryostat sections of the vein were cut at 5 μm and stained using the peroxidase-antiperoxidase method. (×114)

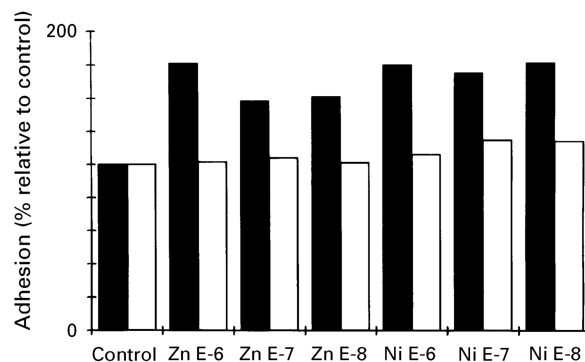

Figure 8 Histogram representation of a typical blocking adhesion assay to quantitate PMN adhesion to an EC monolayer, stimulated for 4 h with zinc and nickel ions at the molar concentrations shown. PMN isolated from human peripheral blood were then allowed to adhere under static conditions for 10 min. The control value was the number of adherent PMN on an untreated EC monolayer. The open histograms show the mean value for PMN adhesion (10 min) after a 1 h treatment of the EC monolayer with a specific anti-E-selectin blocking antibody. The control situation was the PMN adhesion to an EC monolayer pre-treated with an irrelevant antibody. Quantitation was achieved by an automated image analysis system programmed to detect the (small-sized) PMN on the EC monolayer (see Fig. 9).

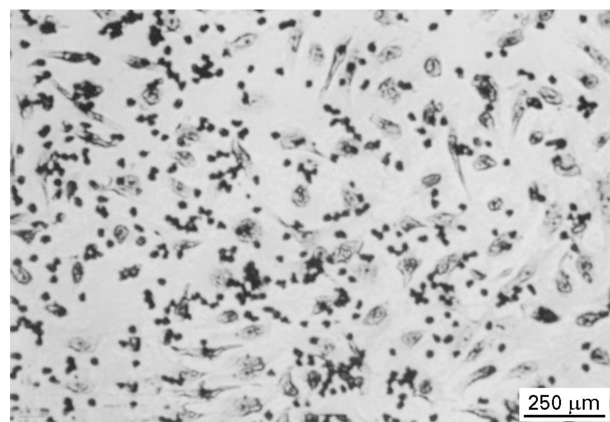

Figure 9 Digitized picture of a random portion of the PMN-EC coculture (10 min adhesion time), following a 4 h exposure of the endothelium to zinc ions at a concentration of E-7 molar.

gene product is absent, and following cytokine stimulation, for example by tumour necrosis factor-alpha (TNF-α), after which the gene product is expressed in abundant amounts [52].

Both ICAM-1 and E-selectin play an essential role in the adhesion of blood leukocytes, especially PMN and macrophages, to EC. Figs. 8 and 9 illustrate an adhesion assay to study how the metal ions, zinc and nickel at concentrations of E-6, E-7 and E-8 molar, can increase the number of PMN adherent to ion-treated EC. The relevance of E-selectin for this increased adhesion is demonstrated by the fact that a specific blocking antibody for this CAM can nullify the metal ion-induced increase in adhesion. In addition to the blocking assay, it is possible to use biochemical methods to show that these metal ions can up-regulate ICAM-1 expression and also induce the expression of E-selectin. In our laboratory we use an enzyme immunoassay (EIA) technique. The metal ion-induced increase in CAM expression is dose-dependent and demonstrates a biphasic reaction pattern, with increased expression at high ion concentrations (e.g. zinc or nickel at E-2 and E-3 M), as well as at very low concentrations (E-8 and E-9 M) [53].

A further level of investigation is to study how the metal ions alter gene transcription, resulting in mRNA production. A suitable method is Northern blot analysis, in which mRNA is extracted from the cultured EC under control and test (i.e. metal ion treatment) conditions, followed by separation in a denatured agarose-formaldehyde gel. This fractionated RNA is then blotted on to a nylon membrane filter and cross-linked to the membrane. With the use of labelled specific c-DNA probes for the CAMs to be detected, it is possible to localize and identify the mRNA of interest by hybridization on the membrane filter. Fig. 10 shows a typical Northern blot to study how cobalt ions in a concentration range from E-3 to E-10 M alter the transcription of ICAM-1 and E-selectin. It can be seen that for ICAM-1 the intensity of the blots in lanes 1 to 8 (decreasing cobalt ion concentrations) are more intense than in the untreated control (lane 9). Lanes 10 and 11 are the positive control conditions, namely

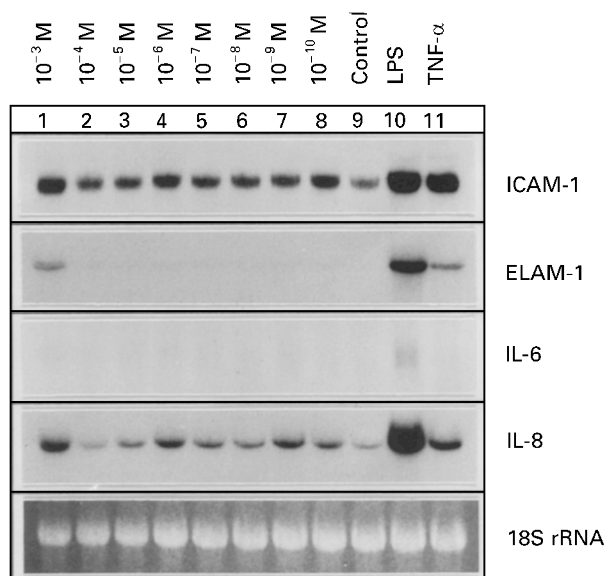

Figure 10 Northern blot analysis of the CAMs, ICAM-1 and E-selectin as well as the interleukins 6 and 8, in EC cultured under negative control conditions (lane 9), positive control conditions (lane 10: endotoxin, 4 μg/ml; lane 11 TNF-α, 300 U/ml) and decreasing concentrations of cobalt ions (E-3 M to E-10 M, lanes 1 to 8). Test conditions involved a 5 h exposure to the given substance. The cDNA probes were α-[32P-d CTP]-labelled by the random prime method. The blots were hybridized first with the specific ICAM-1 probe, followed by stripping with 50% formamide and saline-sodium citrate buffer at 70 °C and reprobing with the specific E-selectin probe. Some of the data presented in this figure are contained in a recent publication in this journal [53].

endotoxin (4 μg/ml) and TNF-α (300 U/ml), respectively. For E-selectin no control signal was given, which acts as an internal negative control, as the E-selectin gene is inactivated in untreated EC. The blot demonstrates that along with the positive controls (endotoxin and TNF-α) only the highest cobalt concentration (E-3 M) is able to induce E-selectin expression. Fig. 11 shows how ISH can be applied to detect mRNA transcripts at single cell level, in this case cultured EC, in which, in the test situation E-selectin was induced by nickel ions.

It should be stressed that with the combination of gene product analysis (e.g. via EIA methods) and gene transcription analysis (e.g. via Northern blotting techniques), it is possible to investigate how various genes are regulated in cells under conditions relevant for biomaterial research, for example, in contact with a given biomaterial surface. These laboratory tools will be of increasing significance in future biomaterial research.

Summarizing this part, it has been shown that cell and molecular biological techniques can be successfully used to understand the pathomechanisms of metal ion-induced inflammation, an important complication of metal implants in the human body.

4. Targeted intervention

Targeted intervention is the accepted approach to treating disease and has as a pre-requisite understanding of the pathomechanisms involved. With respect to the biomaterial field there are two aspects which require addressing, namely biomaterial design and what we will call "supporting measures". The following will discuss the relevance of the cell and molecular biological approach to each of these fields.

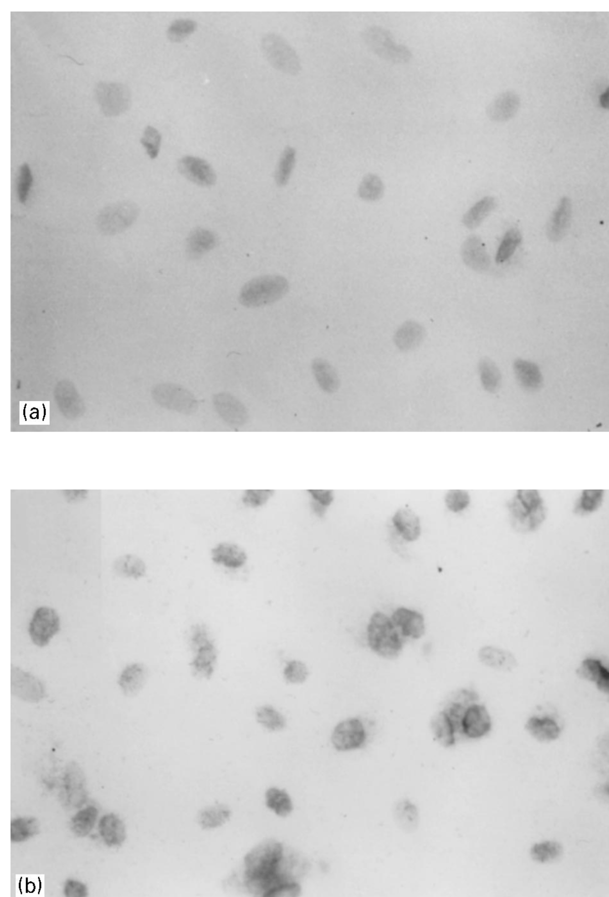

Figure 11 ISH of HUVEC under control conditions (a) and after a 5 h treatment with nickel ions (as chloride) at a concentration of E-3 M (×228) (b). The cells were permeabilized with proteinase K and Triton X-100 after fixation with 4% para-formaldehyde. The ISH technique was used to probe for E-selectin mRNA using digoxigenin-labelled RNA transcripts in an anti-sense sequence. The control, i.e. without nickel ions or using a sense mRNA control, gave no reaction product, indicating the complete down-regulation of E-selectin gene transcription under non-stimulated conditions (a) Nickel ion pre-treatment gave a marked signal in the cytoplasm (b), detected as a dark reaction poduct. (×228)

4.1. Biomaterial design

Our interest in the medical problems associated with the implantation of vascular prostheses for the treatment of advanced atherosclerosis has led us to consider how these prostheses can be improved. This is a necessity, generally recognized [54–56]. One of the major complications of such implants is thrombosis, which results in ischaemia. Based on the biological fact that the endothelium represents the best anti-thrombogenic surface in nature, many research groups have turned their attention to the improvement of vascular prostheses by pre-seeding the luminal surface of the implant with the patient's own EC. This topic has been reviewed elsewhere [57]. This seeding technique is now being applied to microvascular prostheses, that is <2 mm diameter [58].

Three principal approaches have been adopted to improve vascular prosthesis design to promote EC seeding:

Pre-treatment of the prosthesis with adhesion-promoting macromolecules

The theoretical basis for this approach stems from pure cell biology, as it has been shown by numerous authors, that cell adhesion, including that of EC, occurs by recognition via specific cell receptors (especially integrins with the β1-subunit) of macromolecular glycoproteins, such as fibronectin and laminin [59, 60]. Type IV collagen also plays a central role in EC adhesion [61]. That this approach does increase EC adhesion to the inner surface of various types of synthetic prostheses has been demonstrated by several groups using *in vitro* assays and animal experiments [62–65]. Although the first clinical studies in humans indicated that under *in vivo* shear stress long-term patency showed no marked improvement [66], a recent trial on femorocrural bypass presented clear evidence for better early graft patency (first month post-op.) and decrease in amputation rate (at 18 months) [67]. Other research is focusing on the use of more complex basement membrane-like matricies to achieve the same goal of endothelialization [68, 69]. This includes the recently studied combination of elastin-solubilized peptides and collagens type I and III [70], or a gel of collagen type I with dermatan sulphate [71, 72]. It should be stressed that many important questions concerning this approach still remain unanswered, and that empiricism plays a key role in determining the suitability of a particular coating. Much work is still required to understand how the molecular conformation of these macromolecules on the biomaterial surface affects cell adhesion [73].

Surface modification of the prosthetic lumen to create functional or reactive groups

Various studies have shown that relatively simple chemical groups, such as hydroxyl, carbonyl and carboxylic groups, can promote cell adhesion [74–76]. This has resulted in the application of chemical and physical engineering technologies to modify conventional prosthetic materials, such as expanded polytetrafluorethylene (ePTFE) or polyethylene terephthalate (PET), by inserting such functional groups into the most superficial molecular regions of the polymer. Among the technologies are plasma polymerization, glow discharge and radiation-induced grafting [57, 77–80]. The use of pyrolytic carbon coating of ePTFE or PET has been reported to give better growth of EC [81].

Covalent coupling of bioactive molecules

This method is really a hybrid of the first two approaches and is based on the cell biological knowledge that cells recognize relatively small molecular sequences (the so-called "cell-binding regions") within the much larger macromolecules. The prime example is the RGD-sequence (the three amino acids arginine-glycine-aspartic acid), originally described for fibronectin [82, 83], but now known to be a cell-binding sequence in many other adhesion-promoting macromolecules, including vitronectin, fibrinogen, thrombospondin, osteopontin and entactin [84, 85]. The adopted strategy for vascular prosthesis modification has been to covalently couple these oligopeptides (i.e. the bioactive signal molecules) to the luminal surface of the prosthetic polymer [86–89].

The use of cell-binding sequences, such as RGD, immobilized on the surface of a biomaterial is not without possible side-effects. The integrin molecules which are present on the cell membrane and which recognize such sequences are present on numerous cells of different type, so that should the desirable adherent cell become dislodged, the exposed signal molecule could attract and activate an undesirable cell type. The prime example of the latter is the platelet, in the case of endothelial cell-seeded vascular prostheses. Shear stress elicited by flowing blood is most certainly likely to lead to focal loss of cellular coverage. Platelet adhesion and activation form an essential element in the process of thrombosis.

It is evident that such biomaterial design strategies ("tailoring") require fundamental knowledge on *structure-function correlations* for each cell type on a specific biomaterial surface. Fundamental studies on cell interactions with biomaterials are also being carried out for orthopaedic application. Thus, Puleo *et al.* reported the use of Northern blotting and PCR techniques (polymerase chain reaction) to study gene expression of bone-related proteins in osteoblasts in interaction with hydroxyapatite [90].

5. Supporting measures

The principle underlying supporting measures as part of targeted intervention is the possibility of using "*molecular switches*" to control cell function. This could be envisaged not as a permanent therapeutic measure, but as a time-limited intervention at certain critical points in biomaterial application, for example, for a few days after medical device implantation. The corollary to such a strategy is fundamental knowledge of how cells communicate with their surroundings. This involves a study of "*signal transduction*", which is the biological terminology to describe how a cell receives information from its microenvironment and processes it within the cell, leading to a given response. This extremely complex topic in contemporary cell and molecular biology is dealt with in considerable detail elsewhere [91–95]. It is, however, the scientific basis for intervention in undesirable cellular processes, arising as a reaction to biomaterial implantation.

Two main fields of endeavour may be envisaged for the application of molecular switches:

The use of inhibitor substances

Signal transduction pathways represent a series of biochemical reactions which occur sequentially and involve well-defined molecules. Monoclonal antibody

(Mab) technology offers an excellent tool to intervene in these pathways by using a Mab to target an essential component of such a pathway. Other possibilities are the use of ion channel blockers or enzyme inhibitors. Tyrosine kinases are enzymes which phosphorylate proteins during the signal transduction of growth factor action on cells, as well as participate in oncogene activation [96]. Recently, McGregor *et al.* showed that selective inhibitors of tyrosine kinases can reduce cytokine-induced adhesion of granulocytes and monocytes to the endothelium *in vitro* [97].

Application of genetic engineering principles

The present authors are well aware of the intense, and in some cases, heated discussions on the pros and cons of genetic engineering. Nevertheless, this technology will most certainly gain momentum as this century draws to a close, and offers great potential in curbing unwanted effects of biomaterial implantation, even if the present status is still embryonic. Two approaches appear promising:

Control of cell proliferation There are situations in biomaterial application, in which it may be desirable to up-regulate cell proliferation, as, for example, in pre-seeding vascular prostheses with EC. Conversely, inhibition of cell proliferation may be necessary to obviate the negative effects of fibroblast activity following the use of skin substitutes.

Gene product control As for cell proliferation, two opposing actions may be necessary, namely the inhibition of an unwanted cellular product, or, alternatively, the up-regulation of a desirable reaction. A good example of the former principle, and at the same time, of the prospective success of such technologies has been provided by Itoh *et al.* [98]. These researchers were concerned about the control of endothelial production of growth factors. This is very relevant to the anastomotic region between a vascular prosthesis and the remaining natural vascular wall, especially with regard to future developments for small calibre vessels (<6 mm diameter). It also applies, however, to microvascular proliferation in granulation tissue, which is an integral component of wound healing and in some biomaterial applications a serious problem on account of excess scar tissue production. Itoh and colleagues wanted to test the hypothesis that one could use an anti-sense mRNA sequence to block the transcription of an important growth factor, basic fibroblast growth factor (b-FGF), in EC. This anti-sense sequence was an oligonucleotide, complementary to part of the nucleotide sequence in the mRNA of b-FGF (Fig. 12). Their *in vitro* experiments demonstrated that this interventive approach can indeed prevent a gene product from being synthesized, without apparent negative effects for the cell. The latter aspect is the field of endeavour which will require careful scrutiny before *in vivo* intervention can be justified.

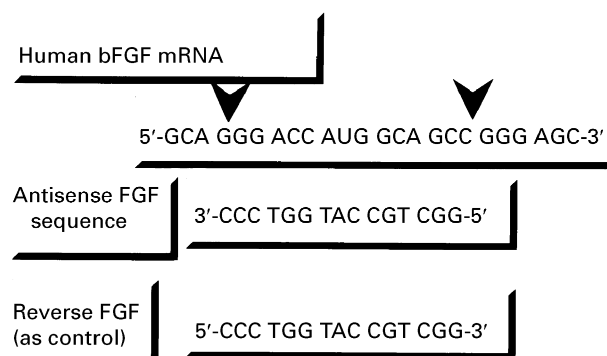

Figure 12 Use of an anti-sense mRNA sequence, complementary to part of the nucleotide sequence of the mRNA encoding b-FGF. Each of the triplet nucleotides, e.g. GCA, GGG etc, codes for a single amino acid in the subsequent translation process of protein synthesis. Itoh *et al.* [98] chose a portion of b-FGF mRNA containing five nucleotide triplets (between arrows). The anti-sense FGF sequence will hybridize to these five triplets and so block the translation process. The control, called reverse FGF is the antisense sequence which has been integrated in the direction 5′ to 3′, instead of 3′ to 5′, i.e. in a reverse direction.

On the topic of up-regulation of a desirable effect, Dichek *et al.* [99] have successfully used retroviral-mediated gene transfer to insert the gene for human tissue-type plasminogen activator into cultured sheep EC. These cells were then successfully seeded on to stainless steel stents. Further studies indicate that genetically engineered EC seeded on to such stents are also to a large extent resistant to pulsatile shear stress [100].

It is encouraging to note that in recent times, various groups in the biomaterial research field have adopted molecular biological techniques to improve understanding of tissue–biomaterial interactions. Thus, Radder *et al.* used Northern blotting methods to study the expression of osteocalcin, osteopontin and β-actin mRNA in the cellular reaction to implanted bone-bonding and non-bonding polyethylene oxide/polybutylene terephthalate copolymers [101]. We feel sure that this trend will continue and that through the application of modern techniques of cell and molecular biology the biomaterials field will continue to progress, in the interest of both patient care and scientific advancement.

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