Meeting Reports

3rd International Workshop on Carcinoma-Associated Mucins, August 7–11 1994, Cambridge, UK

Joyce Taylor-Papadimitriou and Joy Burchell organized a highly successful meeting in a beautiful environment at Robinson College in Cambridge. With energy supplied by outstanding gourmet food, participants focussed mainly on the structures and functions of mucins (in particular MUC1) in health and disease, and on immunotherapy for cancer using immunogens comprising mucin epitopes. Many excellent lectures and posters were presented, and this meeting report is a short summary of selected presentations.

The structural aspects of human mucins were discussed in the first sessions by Young Kim, Mary Rose, John Sheehan and Ingemar Carlstedt. Mucin genes are very large due to the tandem repeat regions. Although it has been a major undertaking to clone and sequence mucin genes, the recent successes in the cloning of larger portions of human mucins MUC1 to 7, rat intestinal and other mucins have led to the eludication of the importance of structural elements of mucins. Young Kim reported that two major mucins, MUC2 and MUC3, are expressed in the human intestines. MUC2 (>5100 amino acids) has a signal sequence characteristic of secreted proteins, heavily O-glycosylated repeat sequences and 4 repetitive elements resembling the D-domain of pre-pro-von Willebrand factor. These regions may be involved in disulfide bonding and mucin 'packaging'. Jim Gum analysed the upstream region of the MUC2 gene and found several regions which may be important in gene regulation. A TATA box and binding sites for numerous transcription factors are present. The MUC2 homologue in rodents shows similarity in this region, suggesting its functional importance. Structurally, MUC3 is very different from MUC2. MUC3 contains epidermal growth factor-like regions, it is produced by a different cell type and may be functionally distinct from MUC2. The highly conserved cysteine residues at the termini of mammalian mucins appear to be important for the formation of oligomeric structures. Cloned frog integumentary mucins also have Cys rich regions with sequence similarities to mammalian mucins and to the D-domain of pre-pro-von Willebrand factor, suggesting similar function (reported by Werner Hoffmann). Thus frog mucins are good models to study mucin assembly. In spite of these cloning efforts, however, the structural and functional aspects of heterogeneous, polymorphic mucins of a mucus gel are still poorly understood.

Great interest was shown in the control of tissue-specific mucin gene expression. Joyce Taylor-Papadimitriou and Tatsuro Irimura discussed the regulation and function of MUC1 which is an epithelial cell membrane bound mucin. Several regulatory elements of the MUC1 gene have been identified and a transgenic mouse expressing MUC1 has been obtained to test which elements are responsible for epithelial cell type expression.

Interesting findings were reported by Sandra Gendler, who obtained knock-out mice which do not express the murine MUC1 (episialin) gene but show no obvious pathology. The lack of the ubiquitous MUC1 was obviously functionally compensated for by the over-expression of other cell surface glycoproteins such as GLYCAM-1. Decay accelerating factor and receptor for hyaluronan-mediated motility and other cell surface glycoproteins involved in cell adhesion were also up-regulated. This mouse model can be used to study the function of MUC1 in mammary tumours.

The tissue- and development-specific expression of the rat sialomucins ASGP1 and ASGP2 was discussed by Kermit Carraway. These two cell surface mucins originate from the same gene through cleavage of a precursor protein leaving a membrane-bound glycoprotein subunit containing N-glycans (ASGP2) and a surface-associated mucin ASGP1. ASGP2 was shown to modulate EGF receptor kinase function and Tyr phosphorylation, and ASGP2 epitopes were found in human breast cancer. This sialomucin complex may therefore be implicated in development and tumour progression.

Sen-Itiroh Hakomori discussed selectin-dependent tumour adhesion. He promotes a concept of tumour metastasis that is based on invasion by tumour cells via the selectin ligands present on metastatic cancer cell surfaces. Selectin-mediated adhesion is normally part of the inflammatory response or lymphocyte homing process, but may also help metastatic cells to cross endothelial cell barriers.

The session comprising methods of structural analysis of mucin carbohydrates and glycopeptides was given by Anne Dell, Ten Feizi, Ken Lloyd and Franz-Georg Hanisch. It is clear that the high sensitivity of modern methods of mass spectrometry offers an ideal approach for the elucidation of structures. Small amounts of oligosaccharides, neoglycolipids or glycopeptides can be analysed by electrospray or liquid secondary ion mass spectrometry. Ken Lloyd was also successful in using high pressure anion exchange chromatography in analysing the carbohydrate content of small amounts of mucin carbohydrate in immunoprecipitates.

Mechanisms of glycosyltransferase action, gene expression, gene regulation and glycosylation of mucins were highlighted by David Joziasse, William Gillespie, Henrik Clausen, Joseph Lau, Nancy Shaper, Tony Hollingsworth and Inka Brockhausen. Great interest was shown in the first enzyme of

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the O-glycan pathway, polypeptide α-GalNAc-transferase. After the cloning of the bovine enzyme by the groups of O. Elhammer and L. Tabak, at least two species of the human enzyme were cloned by Henrik Clausen's group. Based on differences in specificity, DNA sequences and RNA sizes, this enzyme (which acts primarily on Thr residues) must exist as multiple forms of a gene family as is the case for several other glycosyltransferases. A GalNAc-transferase with high activity toward Ser, however, remains to be demonstrated. One of the human forms of the enzyme cloned by Clausen's group has 99% homology to the bovine enzyme. Another cloned enzyme from human placenta has only 44% overall homology (with higher homology in certain regions) and is slightly larger, with a distinct specificity and tissue distribution. The activity present in various cell types was tested by several groups (H. Clausen, T. Hollingsworth, F. Hanisch, I. Brockhausen) using MUC1 peptide substrates which have attracted attention as potential immunogens against cancer. Of the four Thr and two Ser residues in the repeat region of MUC1 only three specific Thr residues (within the Thr–X–X–Pro sequence) become efficiently glycosylated.

Inka Brockhausen reviewed the complex changes in pathways of O-glycosylation in various models of colon and breast cancer. In contrast to N-glycan branching which is often increased in cancer, O-glycan branching is decreased in tumour cells, especially in breast cancer cells. Joy Burchell discussed these model breast cancer cells in more detail. Cells that express the MUC1 epitope have reduced glycosylation, apparently due to a lack of O-glycan branching which tends to expose the underlying peptide.

Tommy Nilsson and Sean Munro presented recent views on the intracellular localization of glycosyltransferases. After several years of searching for a Golgi targeting signal it now appears that the interactions of the transmembrane and adjacent peptide regions of Golgi-localized proteins with Golgi membranes determine the degree of retention. Golgi compartments are functionally distinct but physically connected. Sean Munro emphasized the importance of the partition or distillation effect rather than specific amino acid targeting sequences in maintaining a population of Golgi proteins. This effect may be mediated through interactions between Golgi proteins as well as between proteins and other endogenous Golgi membrane components such as cholesterol and glycolipids.

There are natural and disease–induced immune responses against various epitopes of mucins. A number of reports suggested that antibodies against tumour cell surface mucins or against circulating mucins (probably shed from tumour cell surfaces) may be used as tumour markers for diagnostic purpose. Abnormal epitopes are often exposed in disease and this is the basis for immunotherapy using either mucin peptides, carbohydrate portions or glycopeptides. In general, mucins are less glycosylated in cancer and thus expose peptide (eg MUC1 peptide) and immature (eg Tn and T antigens) or prematurely terminated oligosaccharide chains (eg sialyl-Tn antigen). Several groups (including J. Taylor-Papadimitriou and O. Finn) have promoted immunotherapy using MUC1 tandem repeat peptides since these are exposed in breast cancer and other cancers. Anti MUC1 antibodies occur in a high proportion of breast and ovarian cancer patients (Jo Hilger's group, M. Nuti *et al.*). A response to MUC1 vaccination is not expected to cause autoimmune disease since peptide epitopes are not exposed on normal cells. In addition, cytotoxic lymphocytes from tumour-draining lymph nodes of cancer patients recognize MUC1 repeat region peptides in an MHC unrestricted fashion (O. J. Finn).

Not only a reduction of O-glycosylation but also the appearance of specific structures may be characteristic of highly tumourigenic cells. Sialyl-Lewis^X determinants of human breast cancer and squamous-cell lung carcinomas may be protecting cells from cytotoxic attacks (D. Pettijohn *et al.*). Sialyl-Tn antigen had previously been associated with poor prognosis in colon cancer patients. It appears also that this antigen is present on breast tumours of patients with lower survival (David Miles). Research supported by BIOMIRA (presented by Mark Reddish) has shown some success using sialyl-Tn either linked to KLH, human serum albumin or MUC1 or as a clustered (sialyα2-6GalNAcα-Ser)$_3$ derivative as immunogen. Breast cancer patients tested in a clinical trial all showed a humoral response against sialyl-Tn in spite of the varying degrees of immunosuppression present in these patients. Steven Itzkowitz reported that in tumour challenged rats, immunization with sialyl-Tn-KLH or administration of lymphocytes from rats immunized with sialyl-Tn-KLH (but not their antibodies) increased survival.

George Springer reviewed his long-term investigation using human glycophorin derived immunogens containing T and Tn antigens for the vaccination of breast cancer patients. It is difficult to interpret the data, but there appears to be a statistically significant increase in patient survival, suggesting that this type of immunotherapy is promising although it is not a cure for cancer.

Clearly, there is heterogeneity of mucin epitopes on cancer cells and as yet unpredictable differences between cancer cell types as well as varying responses to immunotherapy. Nevertheless, the approach of immunotherapy using mucin-derived epitopes that are present in higher concentration on cancer cells compared to normal cells offers a more natural therapy than conventional chemotherapy. The future will hopefully reveal the effectiveness of this approach.

Inka Brockhausen

Molecular Cell Biology of Cytokines and Matrix, September 21–23 1994, Cardiff, UK

The British Connective Tissue Society in association with the Biochemical Society Glycobiology Group held their autumn meeting on the 'Molecular Cell Biology of Cytokines and

Matrix' in the School of Molecular and Medical Biosciences at Cardiff between the 21 and 23 September 1994. There were several speakers from overseas in addition to resident British speakers. The meeting attracted 200 participants who were treated to up-to-the-minute data on several aspects of cytokines and matrix.

Hans Kresse discussed the interactions of the small dermatan sulphate proteins decorin and biglycan which are both characterized by leucine-rich repeat motifs and the presence of one or two glycosaminoglycan chains respectively. Both proteoglycans have been shown to interact with fibrillar collagens and bind to the fibrils at similar sites. By using recombinant core protein domains it was concluded that decorin exhibits two independent collagen binding sites. A different domain is involved in receptor-mediated endocytosis of decorin. Interestingly, this domain of the decorin core protein also interacts with the RGD-cell-binding region of fibronectin and this property enables decorin to modulate cell adhesion. In contrast to decorin, the biological roles of biglycan are unclear. It is preferentially located in tissues in the pericellular region and may be involved in the control of cell differentiation. The synthesis of biglycan is strongly stimulated by TGFβ but this activity is inhibited when TGFβ is bound to decorin.

Anne Woods presented evidence for a specific role for the syndecan 4 cell surface heparan sulphate proteoglycan (HSPG) acting synergistically with integrins to promote the adhesion of anchorage-dependent cells. Ligation of $\alpha 5\beta 1$ integrin when cells adhere to a substrate of a proteolytic fragment (105kD) of fibronectin containing the RGD-cell binding site, allows attachment and spreading of primary embryonic fibroblasts, but focal adhesion and stress fibre formation requires additional interaction of cell surface HSPG with heparin-binding fibronectin fragments (Hep II region) or a constituent synthetic peptide WQPPRARITGY. Syndecan 4, but not its close relative syndecan 2, is concentrated in focal adhesions of many cell types, colocalising with vinculin and either $\beta 1$ or $\beta 3$ integrins, depending on the substrate, and thus may be the HSPG which plays a critical role in the development and function of focal adhesions. Recent studies indicate that syndecan 4 is involved in the activation of protein kinase C and *in vitro* studies indicate that a cytoplasmic sequence from syndecan 4, but not syndecan 2, can directly activate PKC and potentiate activation by phospholipid and diolein. In addition, overexpression of syndecan 4 in CHO cells induced flattening and the formation of larger, more numerous focal adhesions than in mock-transfected counterparts.

Gera Neufeld described the 165 amino-acid form of vascular endothelial growth factor ($VEGF_{165}$) an angiogenic growth factor which binds to cell surface receptors on vascular endothelial cells. The biological activity of $VEGF_{165}$ is regulated by cell surface HSPGs and its binding to VEGF receptors is abolished when cell surface heparan sulphates are removed by enzymic digestion. The binding of $VEGF_{165}$ to its receptors is inhibited by short heparin fragments and by platelet factor-4 (PF4). PF4 binds directly to $VEGF_{165}$ but not to the 121 amino-acid form of VEGF ($VEGF_{121}$). PF4 inhibits the binding of $VEGF_{165}$ to the VEGF receptors but not the binding to the same receptors of $VEGF_{121}$. Surprisingly, the mitogenic activity of $VEGF_{121}$ is *inhibited* by PF4, indicating that PF4 can inhibit VEGF signal transduction by an unknown mechanism involving an event that occurs after the growth factor has bound to its signalling receptor.

Alan Rapraeger discussed signalling by the fibroblast growth factors and their regulation by heparan sulphate proteoglycans and heparin. The analysis of specific sulphation patterns of heparin and the use of differentially sized heparin fragments demonstrated that bFGF binding and signalling through its receptor tyrosine kinase is dependent on the complex formed between bFGF and heparan sulphates. The hypothesis was advanced that binding of a single heparan sulphate chain to both bFGF and the bFGF-receptor, is necessary for all aspects of signalling including effects of bFGF on cell migration, morphogenesis and differentiation. In contrast, binding of the chain to bFGF alone may elicit a restricted set of signals that stimulate cell division but not other aspects of typical cellular responses to bFGF. In this latter case, the interaction between the FGF and its receptor is characterised by a much lower affinity, possibly due to a faster off rate for the ligand. Heparin fragments of at least 12 sugar units are required for the activation of bFGF and N-sulphate groups and iduronate-2-sulphates were essential constituents of the 'active sites' in the heparin chain. Evidence was presented for the requirement of some 6-O-sulphate groups (attached to C-6 of $GlcNSO_3$ residues) in the heparin subsite that binds to the receptor.

Michael Klagsbrun reported on his recent studies analysing the structural and biological properties of heparin-binding EGF-like growth factor (HB-EGF). It was shown that HB-EGF exists in two forms, a transmembrane form that is anchored to cells and is capable of stimulating neighbouring cells in a juxtacrine manner, and a secreted form that is a mitogenic peptide 86 amino acids in length. The N-terminal domain of HB-EGF has a stretch of 21 amino acids that constitutes a heparin-binding domain responsible for binding to cell surface HSPGs. Treatment of smooth muscle cells (SMC) with heparitinase or with a 21 amino acid synthetic peptide corresponding to the heparin-binding domain of HB-EGF, inhibits HB-EGF binding to its high affinity receptor and its bioactivity by about 70–80%. The structure of the HB-EGF binding site on heparan sulphate is unknown. HB-EGF is particularly interesting from a clinical perspective because it is a more potent mitogen for SMC than either TGFα or EGF and inhibiting its activity could have therapeutic benefits in controlling restenosis after balloon angioplasty of occluded coronary arteries.

John Heath described the phenotypic consequences of constitutive FGF-4 expression during development of chimaeric mouse embryos. A construct design to produce constitutive expression of FGF-4, where the coding region of FGF-4 is under the control of the mouse phosphoglycerate kinase-1

(PKG-1) promoter, was electroporated into Rosa 11 ES cells. Rosa 11 cells possess a promoter trap construct producing constitutive expression of β-galactosidase which permits the identification of ES cell derived tissues within chimaeras. Rosa 11 ES cell clones exhibiting high level expression of FGF-4 were isolated and used to make chimaeras. Chimaeric embryos exhibit abnormalities of limb and central nervous system (CNS) development suggesting that there are multiple roles of FGF-4 during embryogenesis.

Mark Ferguson spole about the mechanisms underlying scar-free healing in the foetus. Of the many differences between foetal and adult wound healing, the quantity and type of growth factors present in the adult wound differ greatly from that in the foetus. Based on these observations he described experiments whereby antibody neutralization of both TGFβ1 and TGFβ2, or exogenous addition of TGFβ3, resulted in wounds which healed with no detectable scars. Such healing wounds also showed decreases in the monocyte and macrophage profile together with a marked increase in dermal regeneration. Remarkably, there was no diminution in wound strength. TGFβ levels could be manipulated at a variety of organizational levels including antisense oligonucleotides, neutralizing antibodies or prevention of TGFβ activation. This research may well provide a new therapeutic window for reduction of scarring in wounded tissues and acceleration of the healing process.

Frank Luyten described how partially purified extracts from newborn calf articular cartilage were found to induce cartilage and bone when implanted subcutaneously in rats. This activity showed characteristics of bone morphogenetic proteins (BMPs). Degenerate oligonucleotide primer sets derived from the highly conserved carboxy-terminal region of the BMP family were designed and used in RT-PCR reactions with poly(A)+ RNA from articular cartilage as template to determine which BMPs are produced by chondrocytes. Two novel members of the TGFβ superfamily were identified and designated Cartilage-Derived Morphogenetic Protein-1 (CDMP-1), and -2 (CDMP-2). Their C-terminal TGFβ domains are 82% identical, thus defining a novel subfamily most closely related to BMP-5, BMP-6 and osteogenic protein-1. Northern Analyses showed that both genes are predominantly expressed in cartilaginous tissues. *In situ* hybridization and immunostaining of sections from human embroys showed that CDMP-1 was predominantly found at the stage of precartilaginous mesenchymal condensation and throughout the cartilaginous cores of the developing long bones, whereas CDMP-2 expression was restricted to the hypertrophic chondrocytes of ossifying long bone centres. Neither gene was detectable in the axial skeleton during human embryonic development. The cartilage-specific localization pattern of these novel TGFβ superfamily members, which contrasts with the more ubiquitous presence of other BMPs suggests a role for these new proteins in chondrocyte differentiation and growth of long bones.

Gill Murphy described the potential importance of localized breakdown of the extracellular matrix, in the invasiveness of several types of tumour. Cell lines transfected with representative members of the matrix metalloproteinases were compared for their ability to invade a reconstituted basement membrane *in vitro* and to give rise to lung nodules after injection into the tail veins of nude mice. Gelatinase A, but no stromelysin-1 or collagenase, was found to convey a metastatic phenotype on the transfected cells. Studies with specific gelatinase inhibitors and transfection of progelatinase A mutants into the cells indicated that invasiveness was dependent not only on the catalytic activity of the enzyme, but also on the properties determined by the non-catalytic C-terminal domain. To investigate the mechanisms whereby gelatinase A can specifically promote cell invasion through matrigel, the action of the enzyme on individual matrix components and subsequent effects on cell matrix interactions were investigated. Initial studies indicated that the specific cleavage of type IV collagen by gelatinase A modifies cell-adhesion properties mediated by the $\alpha 2\beta 1$ integrin but not by the $\alpha 1\beta 2$ integrin. Hence focal activation of cell-bound gelatinase A and its subsequent action on cell-associated matrix could modulate integrin mediated cell–matrix interactions and consequent cellular invasion of the matrix.

Cay Kielty presented an overview of current understanding of the structural and functional role of fibrillin microfibrils, and described new approaches to elucidate genotype–phenotype relationships in Marfan syndrome. Fibrillin microfibrils clearly bind calcium, and loss of calcium causes major conformational changes within the interbead regions which preclude normal lateral association of fibrillin. Disruption to calcium binding caused by point mutations within EGF-like domains suggest a possible mechanism whereby these mutations might give rise to Marfan syndrome. New data were presented on catabolism of fibrillin monomers and microfibrils by serine proteinases. These degradative processes are implicated in the aetiology of a number of inflammatory connective tissue diseases.

Bjorn R. Olsen described identification of the gene defect causing autosomal recessive chondrodysplasia (*cho*) in mice. Homozygous *cho/cho* mice die at birth with severe abnormalities in the cartilage of limbs, mandible and trachea. Mutant cartilage shows atypically thick, banded collagen fibrils, increased extractability of proteoglycans and extreme lack of mechanical coherence. Bones in the limbs of newborn *cho/cho* have wide flared metaphyses and are only about half the normal length. Thus, the *cho* gene is essential for normal cartilage structure and skeletal development. By linkage analysis the *cho* gene was localized to the *Col 1 11a1* locus and subsequent studies showed that the mutation was the deletion of a single C nucledtide in $\alpha 1$(XI) collagen mRNA about 570 nucleotides downstream of the translation initiation codon. This causes a reading-frame shift and introduces a stop codon seven amino acid codons downstream of the deletion. This exciting discovery provides direct evidence for the importance of type XI collagens in the formation of thin collagen fibrils in cartilage and demonstrates that this 'minor' fibrillar collagen is essential for the normal cohesive properties of cartilage.

Ray Boot-Handford discussed the role of collagen X in endochondral ossification, the process by which long bones grow. Type X collagen is a short chain collagen species synthesized only in growth plate cartilage at the time of matrix mineralization. Mutations in the collagen X gene are associated with a form of dwarfism. The biological mode of action of type X collagen is unclear but direct or indirect effects on cartilage development are apparent. Studies are underway to define other genes whose expression is correlated with growth of long bones.

Martin Humphries described the importance of cell-matrix interactions in connective tissue function. These adhesive events determine cell positioning, modulate tissue-specific gene expression, and control cell multiplication. Furthermore, normal adhesive interactions contribute to the pathogenesis of almost all common human disease. Consequently, the development of agents with the ability to either promote or block adhesion are likely to have widespread clinical utility. In eukaryocytes, most adhesion events require members of the integrin gene receptor family and it is likely that rationally designed inhibitors of integrin function will be useful for treating inflammatory and cardiovascular conditions, regulating immune responses, and preventing tumour metastasis. Integrin receptors frequently recognize short aspartate-containing peptide motifs in matrix ligands, the prototype being the tripeptide RGD. In studies on the binding of $\alpha 4\beta 1$ integrin to fibronectin, Humphries found three active sites in the fibronectin molecule, two of which employ a novel integrin motif XDX′P where X = L or I and X′ = V or A. $\alpha 4\beta 1$ is present on leukocyte surfaces and is essential for extravasation from blood to tissues. In mediating this process, the integrin binds to vascular cell adhesion molecule (VCAM) on the endothelial surface. The key active site in VCAM for $\alpha 4\beta 1$ is the sequence IDPS, closely related to the corresponding sequences in fibronectin but containing P rather than V or A in the recognition motif. Reagents based on the IDPS sequence could be the forerunners of therapeutic molecules for treating inflammation and controlling tumour cell metastasis.

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