Review

Uteroglobin: a novel cytokine?

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Abstract. Blastokinin or uteroglobin (UG) is a steroid-inducible, evolutionarily conserved, multifunctional protein secreted by the mucosal epithelia of virtually all mammals. It is present in the blood and in other body fluids including urine. An antigen immunoreactive to UG antibody is also detectable in the mucosal epithelia of all vertebrates. UG-binding proteins (putative receptor), expressed on several normal and cancer cell types, have been characterized. The human UG gene is mapped to chromosome $11q^{12.2-13.1}$, a region that is frequently rearranged or deleted in many cancers. The generation of UG knockout mice revealed that disruption of this gene causes: (i) severe renal disease due to an abnormal deposition of fibronectin and collagen in the glomeruli; (ii) predisposition to a high incidence of malignancies; and (iii) a lack of polychlorinated biphenyl binding and increased oxygen toxicity in the lungs. The mechanism(s) of UG action is likely to be even more complex as it also functions via a putative receptor-mediated pathway that has not yet been clearly defined. Molecular characterization of the UG receptor and signal transduction via this receptor pathway may show that this protein belongs to a novel cytokine/chemokine family.

Key words. Blastokinin; uteroglobin; CC10; ECM; fibronectin; PLA$_2$; receptor; cytokine.

Introduction

Steroid hormones regulate the expression of many genes. However, only a handful of these proteins has been thoroughly characterized. More than 3 decades ago, the laboratory of Joseph Daniel Jr. in the United States and that of Henning Beier in Germany simultaneously discovered a steroid-inducible secreted protein in the uterus of pregnant rabbits. The former named it blastokinin [1], whereas the latter coined the term uteroglobin (UG) [2]. Convincing evidence suggests that this protein is evolutionarily conserved in all vertebrates, and it is perhaps one of the most potent endogenous immunomodulatory and antiinflammatory agents elaborated by the secretory epithelia of all organs that communicate with the external environment [3]. UG is expressed not only in the uterine endometrium but also by the epithelia of many extrauterine tissues [4], including the thymus, pituitary gland, respiratory and gastrointestinal tracts, pancreas, mammary gland, prostate and seminal vesicle. UG is also present in the blood [5, 6] and in urine [7], although it is not synthesized in the kidney. Since its discovery, this protein has been given numerous names that are based primarily upon the organ in which it is detected or the type of xenobiotics with which it interacts. Thus, UG is also known as progesterone-binding protein [8], Clara cell 10-kDa

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protein [9, 10], urine protein-1 [7, 11–13], polychlorinated biphenyl-binding protein [14] and retinol-binding protein [15].

The UG/Clara cell 10-kDa protein complementary DNAs (cDNAs) from mouse [16, 17], rat [18, 19], hamster [20] and pig [21] have been isolated and characterized. The presence of a UG-like protein in the human uterus [22, 23], lung [24] and prostate [25] have been reported, and the isolation and characterization of the human UG (hUG) cDNA [26], the 5′-promoter region [27] and the complete hUG gene have also been accomplished [28, 29]. The amino acid sequence of nonhuman primate UG has been recently reported [30], and a remarkable sequence similarity between primate and rodent UGs has been demonstrated (fig. 1a). It has also been reported that an antigen, immunoreactive to rabbit UG antibody, is detectable in the wet (mucosal) epithelia of virtually all vertebrates [29] (fig. 1b). This finding suggests that UG is an evolutionarily conserved protein that may have important physiological functions (reviewed in [31, 32]). Although the results of numerous in vitro experiments delineated several biological functions of this protein, its physiological roles, until recently, remained unclear. In order to determine the physiological functions of UG, we [33] and others [34] have performed targeted disruption of the UG gene in embryonic stem (ES) cells and generated UG knockout mice.

In this review, we discuss the structural features of the UG protein, its molecular biology and hormonal regulation of UG expression. We also describe the current status of the UG-binding proteins (putative UG receptor) and provide an overview of our present knowledge on the physiological functions of this protein. Despite our attempt to incorporate a comprehensive list of publications in this review, due to the enormous volume of literature in this field and the limitation of space, important contributions of some of our colleagues may have been omitted, for which we offer our sincere regrets.

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Human  UG  EICPSFQRVIETLLMDTPSSYEAAMELFSPDQDMR      35
Rabbit UG  GICPRFAHVIENLLLGTPSSYETSVKEFEPDDTMK      35
Hare   UG  GICPGFAHVIENLLLGTPSSYGTSLKEFQPDDAMK      35
Rat    UG  DICPGFLQVLEALLLGSESNYEAALKPFNPASDLQ      35
Mouse  UG  DICPGFLQVLEALLMESEGYVASLKPFNPGSDLQ       35
Monkey UG  EICPTFLRVIESLFLDTPSSFEAAMGFFSPDQDMS      35

Human  UG  EAGAQLKKLVDTLPQKPRESIIKLMEKIAQSSLCN      70
Rabbit UG  DAGMQMKKVLDSLPQTTRENIMKLTEKIVKSPLCM      70
Hare   UG  DAGMQMKKVLDTLPQTTRENIIKLTEKIVKSPLCM      70
Rat    UG  NAGTQLKRLVDTLPQETRINIVKLTEKILTSPLCEQDLRV  75
Mouse  UG  NAGTQLKRLVDTLPQETRINIMKLTEKILTSPLCKQDLRF  75
Monkey UG  EAGAQLKKVLDTLPAKARDSIIKLMEKIDKSLLCN      70
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(B)

Structural features of uteroglobin

Uteroglobin is a homodimeric protein in which the 70 amino acid subunits, in an antiparallel orientation, are connected by two disulfide bonds. The primary and quaternary structures of rabbit UG [35–38] were the first to be resolved. X-ray diffraction studies [39–44] revealed the structural features of several crystal forms of rabbit UG. Figure 2A shows a ribbon representation of the crystal structure of dimeric recombinant hUG. Each monomer of this protein is made up of four α helices. The two monomers are held together by two disulfide bonds: one located between Cys-3 and Cys-69′ and the other between Cys-3′ and Cys-69. The work of Umland et al. [45] and that from our own laboratory [46] have delineated the crystal structure of hUG. Figure 2B shows the solvent-accessible molecular surface of the crystal structure of the recombinant hUG molecule.
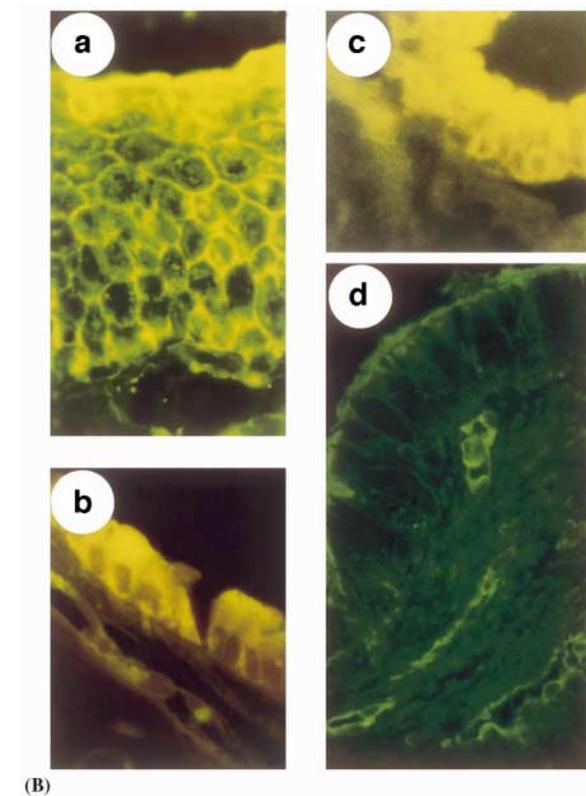
Figure 1. (*A*) Comparison of UG protein sequence among various mammalian species. Note the conserved Cys-3 and Cys-69 residues (boxed) in all species. Also, the 'antiflammin' region in the α-helix-3 is identified by a shaded box. (*B*) Expression of UG-like antigen in various vertebrate phyla. Immunofluorescence of tracheobronchial tissues from different phyla: (*a*) amphibia (frog), (*b*) reptilia (turtle), (*c*) aves (chicken) and (*d*) mammalia (rabbit). (Reprinted with permission from: Zhang Z. et al. (1997) DNA Cell Biol. **16**: 73–83. Copyright © 1998, Mary Ann Liebert, Inc., New York, NY, USA.)

The outer surface of the molecule, exposed to the solvent, is shown in a lighter color. The inner lining of the solvent-exposed molecular surface is shown in magenta. The dimer structure has three cavities: C1, C2 and C3. The cavity, C1, is formed by the two monomers and is made up of mostly hydrophobic residues except for the hydroxyl groups of Tyr-21 and Tyr-21'. This cavity has the volume to accommodate small molecules such as progesterone and retinol. Cavities C2 and C3 are located within each monomer. These cavities are formed by the α helices 1, 2 and 3. While the existence of a large central hydrophobic cavity has been reported [35], the two smaller cavities were not recognized previously. The results of these studies uncovered a remarkable similarity in the structure of UG from humans, rabbit and rat. It is interesting to note that Morize et al. [43] reported a striking similarity in the surface structures of rabbit UG with that of the soluble phospholipase A$_2$ (sPLA$_2$). In fact, these structures are virtually superimposable. While the significance of this structural similarity between these unrelated proteins is unclear, it is to be noted that UG is a potent inhibitor of sPLA$_2$ activity. This is discussed in further detail under 'Biological activities of UG'.

Biological activities of UG

One of the first biological properties of UG to be discovered was its ability to bind progesterone [8], although the physiological significance of this property remains to be elucidated. In addition, UG also binds polychlorinated biphenyls (PCBs) [14] and retinols [15]. Several years ago we proposed that UG may have immunomodulatory properties [47] and as a result may protect the implanting embryo from immunological assault that may be mounted by the maternal organism. Subsequently, in a series of experiments it was demonstrated that UG has potent antiinflammatory and immunomodulatory properties [3, 48, 49]. Levin et al. [50] demonstrated that this protein is a potent inhibitor of PLA$_2$ activity and provided an explanation of how UG may prevent the generation of potent lipid mediators of inflammation (e.g. prostaglandins, leukotrienes etc.). Evidence has amassed in support of the concept that several of its biological properties, including its potent immunomodulatoty [3, 48, 49] and sPLA$_2$-inhibitory [50, 51] activities, may reside in the α-helix-3 of UG [51]. In fact, it has been demonstrated that amino acid residues 39–47 of rabbit UG are well conserved in all species studied so far. Using synthetic peptides corresponding to this region of UG, it has been demonstrated that residues 39–47, at least in part, are responsible for its PLA$_2$-inhibitory activity [51–54]. Because of their potent antiinflammatory and im-

munomodulatory activities, these peptides have been named antiflammins [51], and these effects have been confirmed using several systems [51–71], although some contradictory reports [72, 73] have been published. It has been reported that both intact UG [74] as well as antiflammin-1 [56] also inhibit thrombin-induced platelet aggregation. More recently, it has been reported that antiflammin-1 is a potent inhibitor of chlorpromazine-induced dermal inflammation when this peptide is administered by iontophoresis into guinea pig skin [71]. However, these peptides are unstable and are readily degradable under acidic conditions [75, 76] and this, at least in part, may account for the variable results obtained by some investigators [72, 73].

In order to determine the possible mechanism(s) of inhibition of sPLA$_2$ activity by hUG, Andersson et al. [77] reported that recombinant hUG (rhUG) sequesters Ca^{2+} because it binds calcium. Similarly, using hydrophobic contrast calculations and molecular graphics, Barnes et al. [78] showed a putative Ca^{2+} binding motif in rhUG and suggested this is the structural basis for calcium binding by UG. However, these results should be interpreted with caution for the following reasons: (i) Andersson et al. [77] produced His-tagged UG and used this UG in a PLA$_2$-inhibition assay to demonstrate that UG binds Ca^{2+}, and proposed that this is the reason it inhibits PLA$_2$ activity. It is known that polyhistidine has avidity for calcium ions, and thus if the His tag is not cleaved off the recombinant protein (i.e. rhUG), it may sequester Ca^{2+}, as Andersson et al. have found. Indeed, we were unable to find any statement in the papers of Andersson et al. [77] or in that of Barnes et al. [78] that they have cleaved off the His tag before this protein was used in their PLA$_2$-inhibition assays; (ii) many years ago, when we demonstrated the PLA$_2$-inhibitory activity of rabbit UG, we were concerned that UG might chelate Ca^{2+} and cause sPLA$_2$ inhibition. However, in extensive experiments using natural rabbit UG (purified from uterus), we were unable to demonstrate that this protein binds ^{45}Ca (unpublished results); (iii) while sPLA$_2$ requires millimolar concentrations of Ca^{2+} for its catalytic activity, UG inhibits this enzyme at nanomolar concentrations [50–52, 79, 80] making it highly improbable that the disproportionate stoichiometric ratio between UG and Ca^{2+} depletes this ion sufficiently to cause significant sPLA$_2$ inhibition; (iv) in our laboratory using pure recombinant UG (both rabbit and human), with and without the His tag, we are currently readdressing this question, and our preliminary results show that hUG that does not contain His tag fails to bind Ca^{2+}, contrary to the results of Andersson et al. [77]; (v) our structural analyses of UG by crystallography [46] and by multidimensional nuclear magnetic resonance (NMR) spectroscopy [81] failed to show any Ca^{2+} binding motif in this protein,
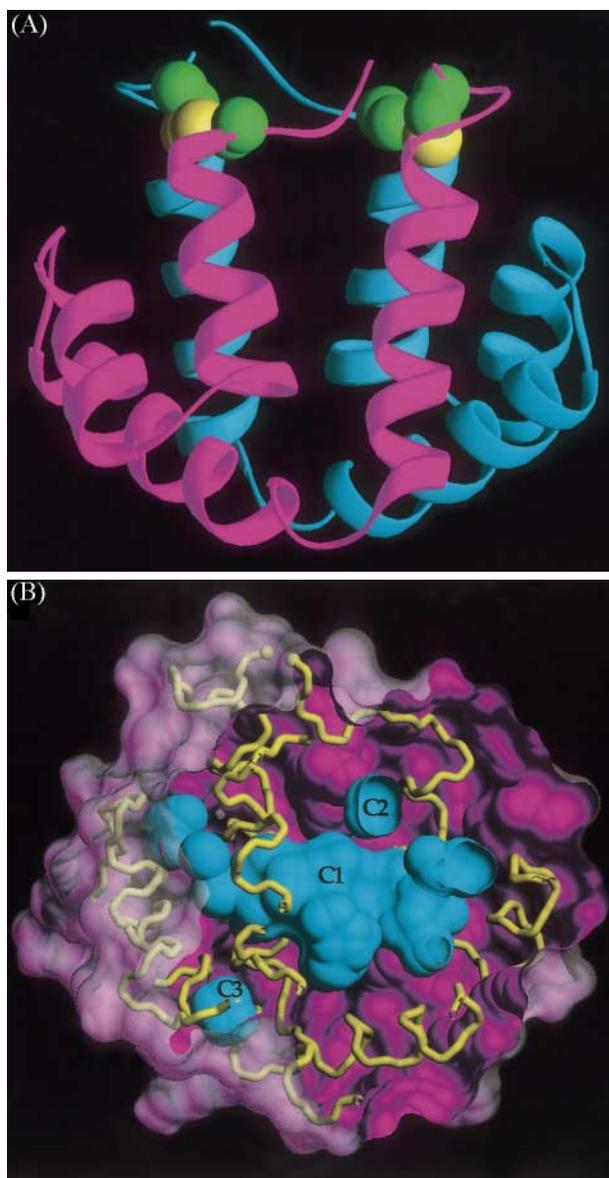
Figure 2. (*A*) Ribbon diagram of the crystal structure of rhUG dimer. Four cysteine residues forming two disulfide bridges are shown as Corey-Pauling-Koltun (CPK) representations. Only the side chain atoms are shown for CYS residues. (*B*) Solvent-accessible molecular surface representation of the crystal structure of the rhUG dimer. The outer molecular surface is represented by the pale color, whereas the cavities are represented by cyan. The cavity labeled as C1 is the largest one formed by the two identical monomers. Two other (symmetric) smaller cavities, C2 and C3, are formed by helix-1, helix-2 and helix-3. For the sake of clarity the front surface of the protein is clipped.

contrary to the results of Barnes et al. [78]. Here again, we could not determine whether the His tag was cleaved off before performing the structural studies. If the His

tag was not cleaved, then it is entirely possible that there was alteration in the quaternary structure of this protein, and this would make interpretation of the results of 'a putative Ca^{2+}-binding motif' more difficult; and finally, (v) site-directed mutagenesis studies in our laboratory have shown that mutation of a single residue (Lys) in a critical region of the UG molecule can abrogate its PLA_2-inhibitory activity (unpublished results), leaving little doubt that the $sPLA_2$-inhibitory property is due to a mechanism other than sequestration of Ca^{2+}.

The electrophoretic mobility of both natural and rhUG/Clara cell protein is anomalous in that it migrates like a 10-kDa protein in SDS-polyacrylamide gel electrophoresis (PAGE), when its calculated molecular mass is 15.8 kDa. This was first observed by Singh et al. [26], who coined the term 'Clara cell 10-kDa (cc10-kDa)' protein, as it was found to be produced by nonciliated epithelial cells in the bronchioles called Clara cells. However, it has been found that other cells in the respiratory tract such as the tracheobronchial epithelia also secrete this protein. Further advances in UG research have been facilitated by the high level expression of recombinant rabbit [79] and human [80] UGs in *Escherichia coli* that allowed comparison of their structural features as determined by X-ray crystallography [46] and by multidimensional NMR [73]. The results of these and other studies showed that rabbit [39–43] and human UGs [45, 46] are indistinguishable proteins both structurally and functionally [79, 80], although there is 68% amino acid sequence identity between these two proteins. A highly allergenic protein, FeldI, found in domestic cats, that has local amino acid sequence similarity to UG, has been described [82]. Recently, a mammary gland protein, mammaglobin, was also found to have some sequence similarity with UG and reported to be overexpressed in human breast cancer cells [83]. Structural similarities of UG with a rat seminal vesicle sperm-binding protein [84], the C2 chain of the rat prostate steroid-binding protein [85], colicin A [86], the CAP domain of haloalkane dehalogenase [87] and lipophilin [88] have been reported. The significance of these sequence similarities has not as yet been clarified, although it has been suggested that these proteins may belong to the UG superfamily [32, 83].

UG cDNA and gene cloning and tissue-specific expression

The rabbit UG protein was first characterized by several groups, and these results facilitated the cloning and characterization of cDNA and the gene [89, 90] encoding this protein. This was followed by the complete nucleotide sequencing of the structural gene for pre-UG [91–96]. The rabbit UG gene spans about 3 kb of DNA
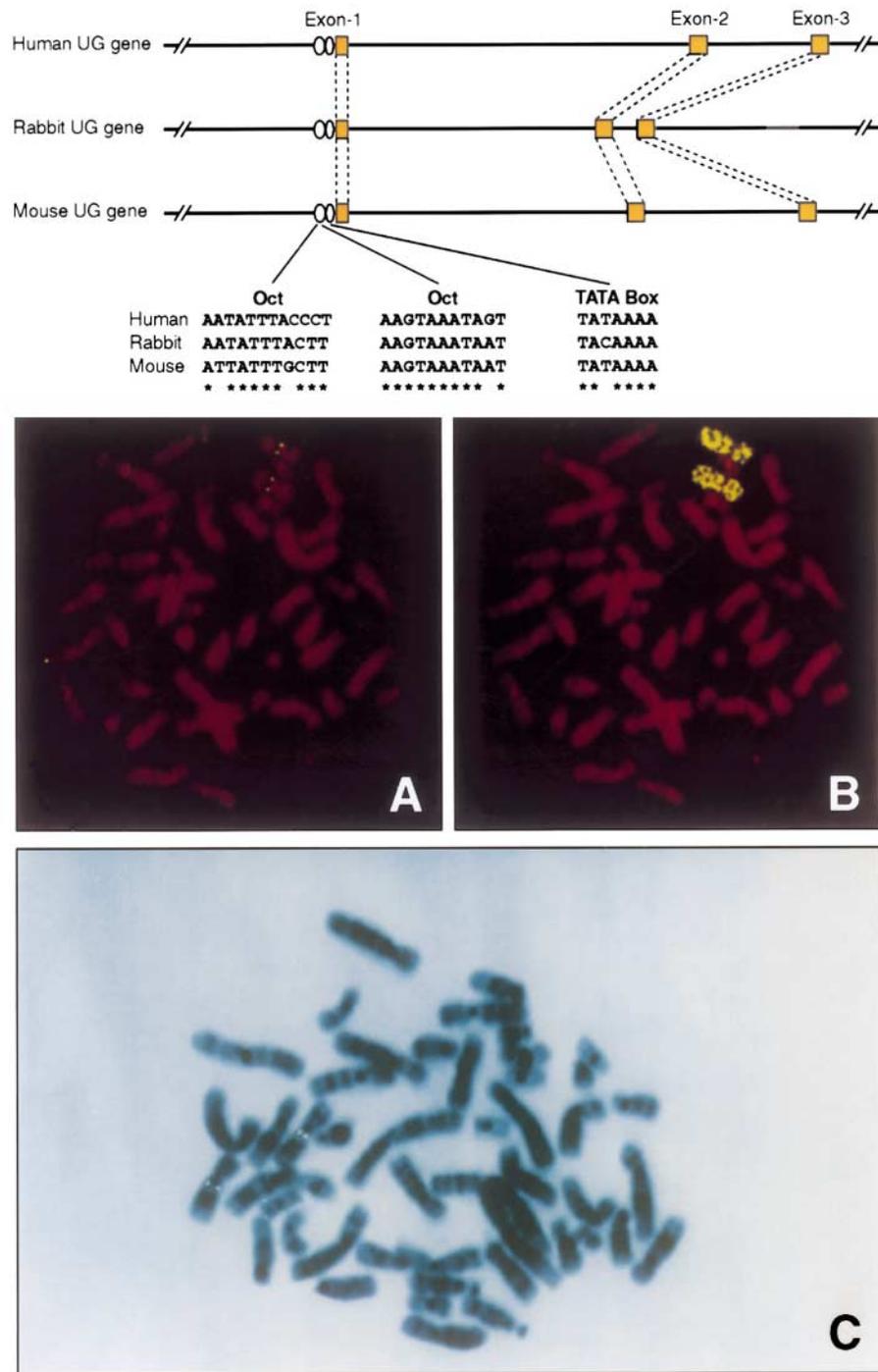
Figure 3. Graphic structural comparison of mouse, rabbit and the human UG genes (upper panel) and chromosomal localization of the human UG gene by fluorescent in situ hybridization (FISH) (lower panel). Upper panel, top rows: Open ellipses represent Oct promoter regions, whereas solid rectangles are the TATA box regions. Upper panel, bottom rows: sequence alignment of homologous regions of human, rabbit and mouse UG genes. Partial Oct promoter and TATA box sequences are shown. Lower panel: (*A*) Digital image of the metaphase chromosomes from a normal donor 46, XY hybridized with a biotin-11-dUTP-labeled hUG genomic probe. Two medium-sized, apparently homologous submetacentric chromosomes have symmetrical fluorescent labeling on the long arms. (*B*) The same metaphase chromosomes after rehybridization with a whole chromosome 11 painting probe. Both labeled chromosomes are identified as chromosome 11. (*C*) Regional FISH localization of the hUG locus at $11q^{12.3-13.1}$ contrast-enhanced, LUT-inverted and digital image of DAPI-banded chromosomes. (Reprinted with permission from: Zhang et al. (1997) DNA Cell Biol. **16:** 73–83. Copyright © 1998, Mary Ann Liebert, Inc., New York, NY, USA.)

and consists of three exons and two introns [94–97]. The 5′-flanking region of the UG gene contains steroid hormone response elements which regulate the steroid-induced, tissue-specific expression of the UG gene [94–97]. Several years later, the human UG cDNA [26] and subsequently, the human UG gene were cloned and characterized [28, 29]. The UG gene structures are remarkably similar among human, rabbit and mouse (fig. 3, upper panel). The location of the UG gene has been mapped [29] to human chromosome $11q^{12.3-13.1}$ (fig. 3, lower panel), a region in which many candidate disease genes have been mapped by linkage analyses. Furthermore, the presence of an antigen, immunoreactive to UG antibody, is detectable in all vertebrate phyla [29], further suggesting the evolutionary conservation and importance of this protein.

Although the original discovery of this protein was made in the rabbit uterus during early pregnancy [1, 2], investigations that followed revealed that in addition to the uterus the UG gene is also expressed in numerous other organs [98–108]. The presence of UG in humans was initially the subject of conflicting reports [109–112]; however, subsequent studies confirmed its presence in the human endometrial [22], tracheobronchial [24] and prostatic [25] epithelia by Western blotting and immunohistochemistry. Furthermore, expression of UG in human uterus was also found to be regulated by progesterone and estrogen [22].

UG gene regulation by hormones and other agents

Regulation of UG gene expression by steroid hormones (e.g. estrogen and progesterone) was experimentally demonstrated by in vitro translation and nucleic acid hybridization. These results suggested that both estradiol and progesterone profoundly affected the expression of the UG messenger RNA (mRNA) in rabbit [113–118] as well as human [119] endometrium. It was also shown that estradiol increases the level of UG mRNA by 4-fold, whereas a sequential administration of estradiol and progesterone to the rabbit increased it 12-fold [116]. This effect was not due to the stability of the UG mRNA. Recently, it has been reported that progesterone-induced UG gene expression is further augmented by prolactin [120, 121]. This nonsteroid hormone appears to enhance progesterone-induced UG gene expression in the uterus [122–127]. Moreover, it is now clear that prolactin transcriptionally augments progesterone-induced UG gene expression in the uterus via a specific protein that binds to the 5′-flanking region of the UG gene [124–126, 128]. In the prostate and in the seminal vesicle, the UG gene is regulated by testosterone [129], whereas in the lungs it is constitutively expressed, although glucocorticoid treatment caused a three-fold stimulation of UG mRNA expression in the rabbit [130–132]. Differential activation of lung-specific

CC10 (UG) by two forkhead proteins, FREAC-1 and FREAC-2, has been reported by Hellqvist et al. [133]. Recently, it has been reported that administration of IFNγ to mice stimulates UG production in the lungs, and an interferon (IFN)γ-response element has been identified in the 5′-flanking region of the mouse UG promoter [134]. Interestingly, the production of IFN-γ and its biological activity are also inhibited by UG [135, 136]. More recently, it has been demonstrated that IFN-γ treatment of human bronchial cells induces expression of UG [137]. Taken together, these results further suggest that UG has an important role in the regulation of immunological and inflammatory processes. Whether these effects of UG are mediated by the UG receptor is yet to be determined.

Multifunctional nature of UG

We mentioned earlier in this review that UG has been given numerous names. These names may suggest the multiple biological properties of this protein. One of the first properties reported was its ability to enhance the growth of preimplantation blastocysts, hence the name blastokinin [1]. Subsequently, it was found that UG binds progesterone [8], and it was proposed that UG acts as a carrier and/or scavenger [138] of this steroid hormone, reducing its toxicity to the early developing embryo [139]. Several years later, binding of PCBs [14] and retinol [15] by UG was also reported. The significance of these results needs to be clarified.

In 1980, Mukherjee et al. [47] proposed that UG may provide immunological protection to the developing embryo, which is an allograft to the maternal organism. Subsequently, experimental evidence was provided which, at least in part, lent support to this hypothesis [48, 49]. Furthermore, UG was suggested to be one of the mediators of pregnancy-related immunosuppressive effects of progesterone, which Siiteri et al. [140] called 'Nature's immunosuppressant'. Interestingly, the immunomodulatory effects of UG were found to be further augmented by transglutaminase (TG), an enzyme that catalyzes the cross-linking of proteins by forming ε-γ-glutamyl-lysine isopeptide bonds between lysine and glutamine residues in them. In fact, further experiments showed that UG is an excellent substrate of TG [71, 72, 141]. One of the most important properties of UG is its ability to dramatically inhibit both chemotaxis and phagocytosis of monocytes and neutrophils [142, 143]. Taken together, these results raised the possibility that UG alters the immune response against allogenic cells by masking their surface antigens and directly modulating phagocyte functions. Recently, this protein has again been called a 'natural immunosuppressor' [136]. These results, in conjunction with the recent discovery

that UG has a cell surface receptor [144–146], raise the possibility that UG may be a multifunctional cytokine. The cloning and characterization of the UG receptor cDNA and the gene may further advance our knowledge of the function of this protein.

A well-known dictum in reproductive biology is that progesterone induces quiescence in the mammalian uterus during pregnancy and that successful pregnancy in all mammals requires progesterone. It is also known that the motility and contraction of the uterine smooth muscles are induced by lipid mediators of inflammation (e.g. prostanoids). The production of prostanoids (PGs) requires arachidonic acid, generated by the hydrolysis of cellular phospholipids by PLA_2s, a diverse family of acyl esterases [147–151]. Since a family of corticosteroid-induced proteins, lipocortins [152–155], exert their biological effects by inhibiting PLA_2 activity, the possibility that UG may function in a similar manner was investigated. It was found that UG is a potent inhibitor [50, 51] of $sPLA_2$ activity. This function appears to be conserved in UG and can be attributed to amino acid residues 39–47 of the α-helix-3 of UG [51] in virtually all mammals [29].

It is interesting that UG is constitutively expressed in the tracheobronchial epithelia that comes in contact with myriads of antigens that are present in the external environment. It is proposed that in this organ UG may function as a modulator of inadvertent immunological activation [3]. In addition, UG may have another very important function, that is inhibition of proliferation of certain cell types, and this effect may be receptor-mediated. Recent evidence suggests that the epithelia in organs that under physiological conditions express the UG gene may not do so after they undergo transformation [156–158]. Furthermore, isolated epithelial cells from those organs when transformed by an oncogenic virus (e.g. SV40) show drastic reduction in UG gene expression or do not produce UG at all [156, 157]. These results show that UG production is not compatible with the transformed phenotype of a cell. Most interestingly, induced expression of UG in some cancer cells appears to result in the loss of their transformed phenotype [158] that typifies these cells. In addition, the cancer cells that respond to induced UG expression by losing their malignant transformed phenotype also appear to express the UG receptor (Z. Zhang et al., unpublished results). These results not only define the multifunctional nature of this protein but also raise the possibility that UG may have a tumor suppressor-like function. Most important, our preliminary studies involving UG-knockout mice suggest that the incidence of cancer in these mice is extremely high (Z. Zhang et al., unpublished results). Taken together, these results point to the tumor suppressor-like activity of UG.

Uteroglobin-binding proteins (putative receptor)

Daniel and his colleague [1] and Kirchner [100, 101] independently observed that whereas UG is synthesized and secreted by the endometrial epithelia of the rabbit, this protein was detectable in the blastocoele cavity. However, neither how UG is transported across the trophoblast layer of the blastocyst nor the physiological function of UG in the blastocoele have been determined. Robinson et al. [159] first proposed that a transporter may be involved in migration of UG from the uterus to the blastocoele. Using ^{125}I-hUG (recombinant) as the ligand, we found high-affinity UG-binding protein (putative receptor) on several cell types with a molecular mass of 190 kDa [145]. Diaz Gonzalez and Nieto [144] reported specific binding of ^{125}I-UG to proteins on microsomal and plasma membranes. Since UG is a homodimer in which the 70-amino acid subunits are connected by two disulfide bonds, we sought to determine whether UG monomers also interact with the 190-kDa UG-binding protein and, if so, whether the UG monomer has the same biological activity as the dimer. Surprisingly, we uncovered an additional protein, with a molecular mass of 49 kDa, that binds reduced UG with high affinity and specificity [146]. Both 49- and 190-kDa proteins (fig. 4) are readily detectable on nontransformed NIH 3T3 and some murine cancer cells (e.g. mastocytoma, sarcoma and lymphoma), while lacking on others (e.g. fibrosarcoma). Most interestingly, UG pretreatment of the cells express the binding proteins,
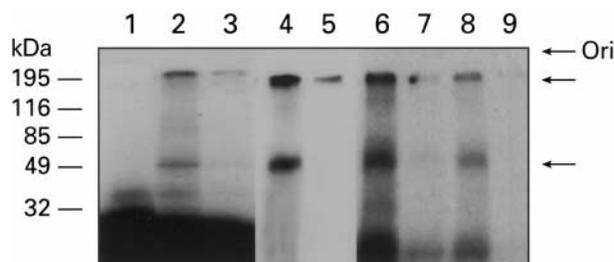

Figure 4. Affinity cross-linking of hUG-binding proteins on NIH 3T3 (lanes 1–3), mastocytoma (lanes 4–5), sarcoma (lanes 6–7) and lymphoma (lanes 8–9) cells. ^{125}I-hUG was incubated with each of these cells in the absence or presence of unlabeled hUG for binding and then cross-linked with disuccinimidyl suberate (DSS). Lane 1: (−) DSS; lane 2: (+) DSS; lane 3: (+) unlabeled hUG, (+) DSS; lane 4: (+) DSS; lane 5: (+) unlabeled hUG, (+) DSS; lane 6: (+) DSS; lane 7: (+) unlabeled hUG, (+) DSS; lane 8: (+) DSS and lane 9: (+) unlabeled hUG, (+) DSS. Note a 49-kDa protein band, in addition to the 190-kDa band and the decreased intensity of both bands when nonradioactive UG was added to the reaction mixture for competition. (Reprinted with permission from: Kundu G. et al. (1998) J. Biol. Chem. **273:** 22819–22824. Copyright © 1998, The American Society for Biochemistry and Molecular Biology.)

dramatically suppressed extracellular matrix (ECM) invasion, though such treatment had no effect on fibrosarcoma cells that also lack the binding proteins. Tissue-specific expression studies confirmed that UG-binding proteins are present in bovine heart, spleen, lung, liver and the kidney but not in the aorta (fig. 5A). Purification of these binding proteins from bovine spleen by UG-affinity chromatography and analysis by SDS-PAGE followed by silver staining identified a 40- and an 180-kDa protein (fig. 5B), respectively. Treatment of the NIH 3T3 cells with specific cytokines (i.e. IL-6) and other agonists [i.e. lipopolysaccharide (LPS)] substantially increased ^{125}I-UG binding on these cells but pretreatment with PDGF, TNFα, IFNγ and phorbol myristate acetate (PMA) was ineffective (fig. 6). These results suggest that (i) two proteins are readily detectable on both normal and malignant cells that bind UG with high affinity and specificity; (ii) these proteins are also detectable in bovine heart, lung, spleen and kidney but not the aorta; (iii) treatment of the NIH 3T3 cells with LPS or IL-6 substantially increases the level of ^{125}I-UG binding and (iv) UG pretreatment of the cells, expressing the high-affinity binding proteins, causes dramatic inhibition of ECM invasion, whereas it has no such effect on cells lacking UG-binding proteins [145, 146]. Taken together, these findings raise the possibility that UG-binding proteins play critical roles in UG-mediated regulation of cellular motility and invasion of the ECM.

Determination of physiological functions of uteroglobin by gene targeting in mice

The properties of UG described so far have been derived primarily from the results of in vitro experiments. While these investigations uncovered important biological properties of this protein (for review see [3, 31, 32, 160]), more than 3 decades of scientific inquiry did not uncover the physiological functions of UG. Thus, it was necessary to inactivate the UG gene to generate an animal model lacking this protein. Accordingly, the UG gene was disrupted in mice [33] by gene targeting [161] in ES cells. The resulting phenotype
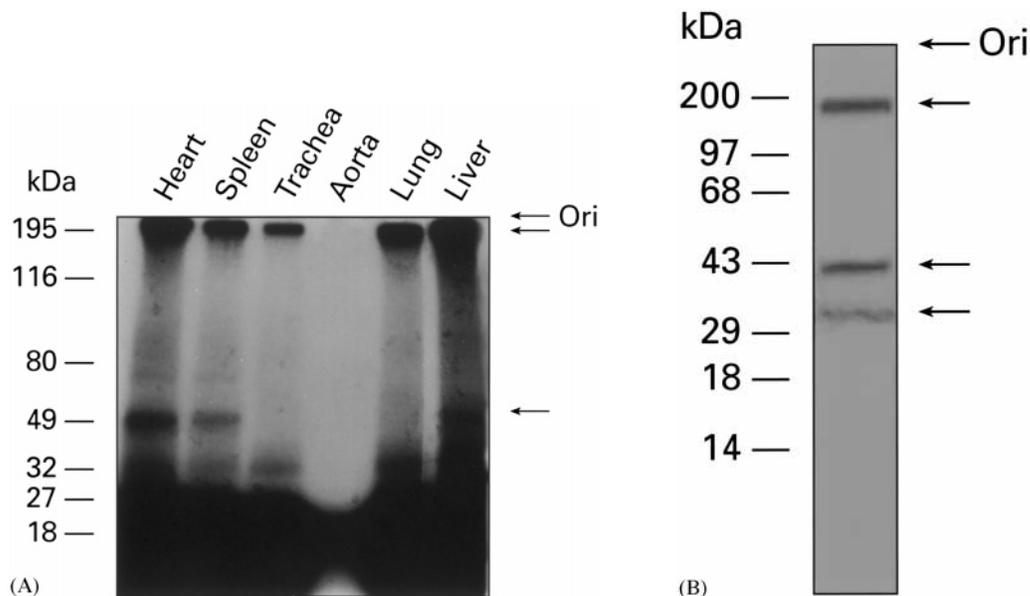

Figure 5. (*A*) Tissue-specific expression of hUG-binding proteins using different bovine tissues by affinity cross-linking technique. Note that the hUG-binding proteins are clearly detectable in bovine heart, spleen, trachea, lung and liver but not in the aorta. (Reprinted with permission from: Kundu G. C. et al. (1998) J. Biol. Chem. **273**: 22819–22824. Copyright © 1998, The American Society for Biochemistry and Molecular Biology.) (*B*) Affinity purification of UG-binding protein(s). The UG-binding proteins were purified using UG affinity chromatography. The purified receptor proteins were resolved by SDS-PAGE under denaturing and reducing conditions, and the protein bands were visualized by silver staining. Note that there are two protein bands with apparent molecular masses of 180- and 40-kDa, respectively, that are clearly visible. In addition, a third faint band with an apparent molecular mass of 32 kDa is also detectable. However, unlike the 180- and 40-kDa proteins, the 32-kDa species had no UG binding (data not shown), suggesting that this band is either an artifact or a degradation product that lack the UG-binding epitope. (Reprinted with permission from: Kundu G. C. et al. (1998) J. Biol. Chem. **273**: 22819–22824. Copyright © 1998, The American Society for Biochemistry and Molecular Biology.)
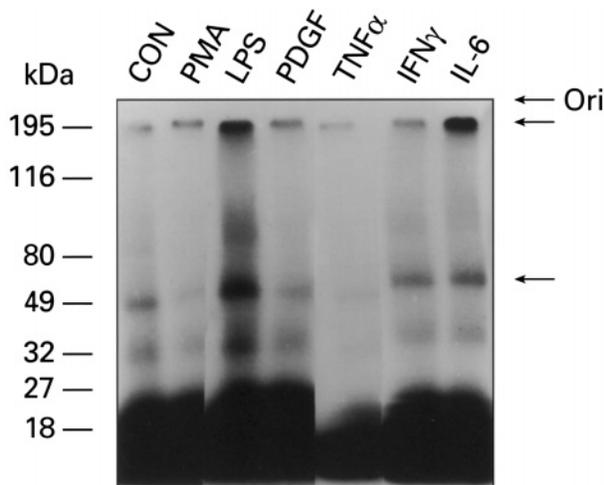
Figure 6. Effect of different cytokines and other agents on the expression of UG-binding proteins using NIH 3T3 cells. Note the considerable enhancement in intensity of the radioactive bands representing the UG-binding protein(s) following treatment of the cells with LPS and IL-6, respectively, compared with the control. However, this difference is not apparent when the cells are treated with PMA, PDGF, TNFα and IFNγ. (Reprinted with permission from: Kundu G. C. et al. (1998) J. Biol. Chem. **273**: 22819–22824. Copyright © 1998, The American Society for Biochemistry and Molecular Biology.)

from one of these studies [33] has recently been reviewed [162, 163]. Thus, in this review we will summarize our findings and discuss the implications of the phenotype of the UG knockout mice, especially the development of fibronectin (Fn)-deposit glomerulopathy [33] in these animals.

Targeting and disruption of the UG gene were accomplished by partial deletion of exon-2 and by inserting a neomycin resistance gene cassette (*neo*) as shown in figure 7A. Standard techniques [161] were employed to generate UG-deficient mice [33]. The resulting targeting construct was transfected to the ES cells. Figure 7B shows a Southern blot of genomic DNA from ES cell clones in which the UG gene has been successfully disrupted. In figure 7C and D, the polymerase chain reaction (PCR) analyses and Southern blotting, respectively, of the genomic DNA from UG gene-targeted offspring are shown. The UG deficiency was verified both by reverse-transcriptase PCR (RT-PCR) (fig. 7E) and Western blotting (fig. 7F) using lung RNA and protein, respectively. We used the lung RNA, as this gene is constitutively expressed in this organ. These results were further confirmed by immunohistochemical analyses of the tissues (fig. 7G). The UG gene-disrupted mice were used to analyze the phenotype manifested as a result of this mutation.

The major phenotypic finding in the UG knockout mice was in the kidneys [33]. This was unexpected, as UG gene is not reported to be expressed in this organ. Compared with normal controls (fig. 8A), the UG knockout mice developed severe glomerulopathy (fig. 8B), either 4–5 weeks after birth (early onset) or when they were several months old (late onset). The animals with the late-onset disease, in addition to developing the Fn-deposit glomerulopathy, had renal parenchymal fibrosis and distal tubular hyperplasia (fig. 8C, D). The glomeruli of the early onset mice demonstrated abnormal deposition of predominantly Fn and a moderate amount of collagen as demonstrated by the presence of characteristic Fn and collagen fibrils by electron microscopy (fig. 8E, F). These results were confirmed by immunofluorescence and Mason's trichrome staining, respectively (fig. 8G–J). Biochemically, these Fn molecules appeared to be multimeric when analyzed by SDS-PAGE (fig. 9A). In order to determine whether excessive production of Fn may have resulted in abnormal glomerular deposition, we performed semiquantitative RT-PCR and densitometric analyses of the cDNA bands obtained by using total RNA from whole kidneys, lungs and liver of UG$^{-/-}$ and UG$^{+/+}$ mice, respectively. Although these experiments did not show that Fn production was higher in UG$^{-/-}$ mice, semiquantitative RT-PCR experiments using RNA from isolated glomeruli of UG null and control mice now show that Fn production in the glomeruli of UG null mice is elevated (F. Zheng et al., unpublished results). The development of glomerulonephritis, an inflammatory disease [164], in UG gene knockout mice is not surprising, as UG is an antiinflammatory protein. However, the development of an inflammatory disease in the kidney was fully unexpected, as this organ does not express the UG gene.

As mentioned earlier in this review, UG is a potent inhibitor of sPLA$_2$ activity [17, 50–52]. Thus, it was important to determine the plasma PLA$_2$ activities in UG$^{-/-}$ and UG$^{+/+}$ mice, and we found that the specific activities were significantly higher in UG$^{-/-}$ mice compared with UG$^{+/-}$ or UG$^{+/+}$ controls. We also found that lysophosphatidic acid (LPA), a byproduct of PLA$_2$ catalysis, was also elevated in the plasma of UG-deficient mice. The implications of these findings will be clear in the following paragraphs. A high molecular ECM protein, Fn [165–167] is known to interact with several other polypeptides. According to the current concepts, during matrix assembly and fibrillogenesis, self-aggregation of Fn and activation of integrins [165–167], heterodimeric proteins with receptor-like functions, play critical roles. Fn predominantly binds to $\alpha_5\beta_1$ integrin present on the surface of several cell types, including glomerular messangial cells. Recent reports also indicate that integrin activation and
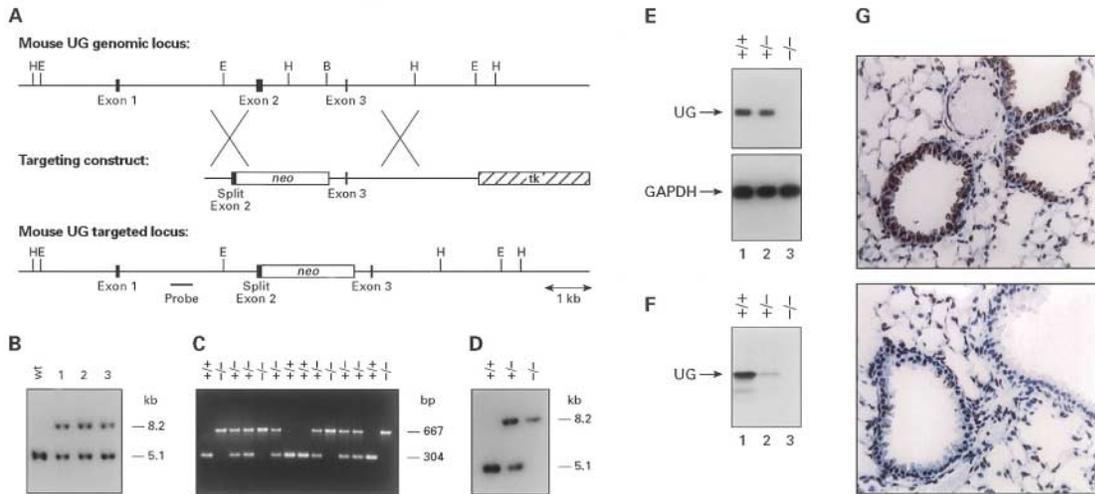
Figure 7. Generation of UG-knockout mice by gene targeting. (*A*) Schematic representation of the UG gene locus is shown in the upper panel. The restriction sites are identified by single letters (B, *BamHI*, E, *EcoRI*, H, *HindIII*). (*B*) Southern blot analyses of the targeted ES R1 cell clones. *Wt*, wild type. (*C*) Representative PCR analyses of genomic DNA from tail biopsies of offspring. The genotypes and their corresponding PCR products are as follows: UG$^{+/+}$, 304 bp; UG$^{+/-}$, 304 and 667 bp; UG$^{-/-}$, 667 bp. (*D*) Southern blot of mouse tail genomic DNA. (*E*) RT-PCR analyses of total RNA extracted from the lung tissues of littermates with UG$^{+/+}$, UG$^{+/-}$ and UG$^{-/-}$ genotypes. Whereas a 273-bp RT-PCR product was clearly detectable in the lungs of UG$^{+/+}$ and UG$^{+/-}$ mice, it was lacking in those from UG$^{-/-}$ mice. (*F*) Western blot analysis. Proteins (30 μg each) from lung lysates were resolved by electrophoresis using 4–20% gradient SDS-polyacrylamide gels under nonreducing conditions and immunoblotted using rabbit anti-mouse UG. (*G*) Immunohistochemical localization of UG in bronchiolar epithelial cells. The dark staining over the bronchiolar epithelial cells of a UG$^{+/+}$ mouse (upper panel) indicates the presence of UG immunoreactivity. Note a complete lack of such immunoreactivity in the lungs of the UG$^{-/-}$ mouse (lower panel). (Reprinted with permission from: Zhang Z. et al. (1997) Science **276:** 1408–1412. Copyright © 1998, American Association for the Advancement of Science.)

self-assembly of Fn are promoted by LPA. Thus, we studied whether UG may have an effect on Fn self-aggregation. We found that UG binds to Fn with high affinity, and the formation of Fn-UG heteromer effectively prevents Fn-Fn homomer formation (fig. 9E). Similarly, Fn-collagen aggregation was also prevented by UG (fig. 9F). Furthermore, we have found the presence of Fn-UG heteromers in plasma of UG$^{+/+}$ but not in those of UG$^{-/-}$ mice (fig. 9D). Moreover, in vivo we demonstrated that Fn deposition in the glomeruli of UG$^{-/-}$ mice is undetectable when Fn is infused in combination with UG, whereas infusion of Fn alone can easily cause abnormal deposition [33].

Taken together, our results, in conjunction with the current knowledge of Fn matrix assembly and fibrillogenesis, suggest that one of the important physiological roles of UG is to prevent abnormal deposition of Fn, which leads to the glomerulopathy observed in UG gene-disrupted mice. It should be noted that UG gene knockout mice described by Stripp et al. [34] did not develop the same phenotype as that of the UG knockout mice [33]. While the exact reason(s) for this phenotypic difference are not yet clearly understood, at least in a some instances (for review see [168]) such differences are caused by inadvertent disruption of a gene flanking the gene intended to be disrupted.

Transgenic mice expressing antisense UG-mRNA manifest abnormal deposition of fibronectin and collagen in the renal glomeruli

As discussed above, targeted disruption of the murine UG gene in two independent laboratories manifested two different phenotypes. Phenotypic differences may arise from (i) unintended disruption of genes flanking a target gene (e.g. UG), (ii) genetic background or (iii) production of truncated or fusion gene products as a result of incomplete disruption of the gene. Therefore, in order to address the first possibility, we generated two independent lines of transgenic mice that express antisense UG mRNA-caused partial UG deficiency, without the structural alteration of the endogenous gene. We found that these transgenic mice develop abnormal glomerular deposition of Fn and collagen (G.

Kundu et al., unpublished results) virtually identical to that found in the UG knockout mice [33]. These results strongly suggest that the cause of the renal disease in UG gene knockout mice reported previously [33] is due to UG deficiency caused by UG gene disruption, and it seems highly unlikely that the observed phenotype of these mice is due to the disruption of any other gene flanking the UG locus.

Tumor suppressor-like effects of UG

As indicated earlier in this review, the UG gene, mapped to human chromosome $11q^{12.2-13.1}$ [29], consists of three exons and two introns and was structurally conserved during evolution. Interestingly, abnormalities in this region of chromosome 11 have been correlated with human cancers [169–172]. It has also been reported that the introduction of chromosome 11 into HeLa cells completely suppresses the tumorigenic phenotype of these cells [173]. Recent reports indicate that whereas the UG gene is constitutively expressed in the tracheobronchial epithelia at a high level [3], both adenocarcinoma tissues as well as the cell lines derived from adenocarcinomas of the lung express the UG gene either at a drastically reduced level or do not express it at all [158, 174–176]. The loss of UG gene expression has also been reported in adenocarcinomas of the prostate [177], another organ in which the UG gene is constitutively expressed at a high level [3]. Furthermore, immortalization of normal epithelial cells from rabbit lung (K. Momoeda et al., unpublished results), uterus [156] and prostate (A. B. Mukherjee et al., unpublished results), by an SV40 mutant virus, causes the cessation of UG gene expression. Moreover, in some cell types (e.g. arterial smooth muscle cells) UG treatment inhibits proliferation (G. Mantile-Selvaggi, et al., unpublished results). Taken together, these results suggest that the lack of UG gene expression may be a characteristic shared by many cancer and transformed cells. By transfecting several adenocarcinoma cell lines with hUG-cDNA, we demonstrated that induced hUG expression reverses the transformed phenotype (i.e. anchorage-independent growth and ECM invasion) of only those cells that also express the hUG receptor. Treatment of the receptor-positive cells with purified hUG yields identical results. These data define both autocrine and paracrine pathways through which hUG exerts its effects that reverse the transformed phenotype of cancer cells that carry its receptor.

Is UG a cytokine?

This question arises with regard to UG for several reasons: (i) like cytokines, UG is a secreted protein; (ii)
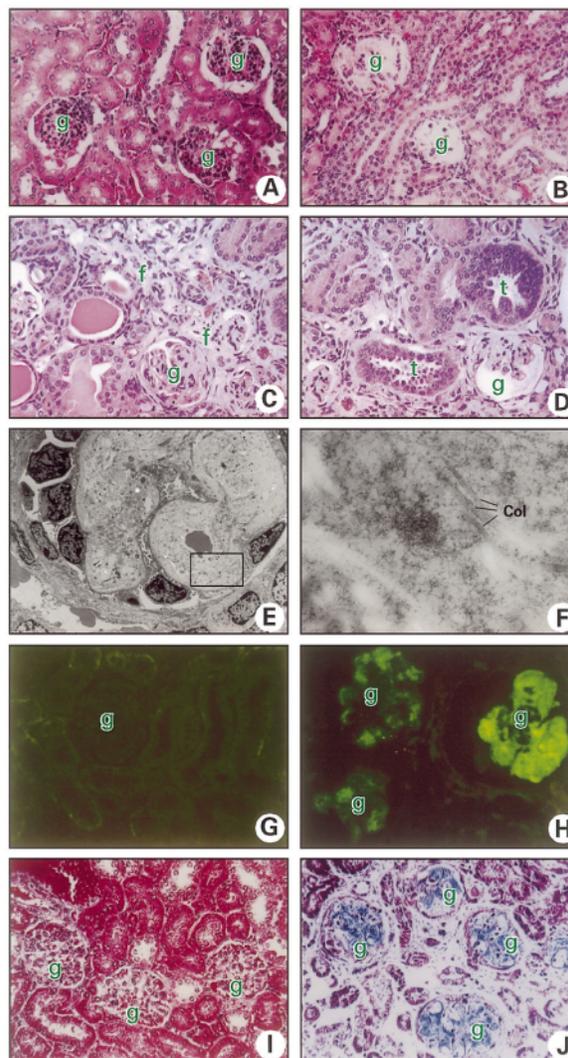

Figure 8.   Severe renal glomerular disease in $UG^{-/-}$ mice. H&E staining of kidney sections from a $UG^{+/+}$. (*A*) and its $UG^{-/-}$ littermate (*B*). Note the heavy deposit of eosinophilic material in the glomeruli of the $UG^{-/-}$ mouse with severe renal disease. (*C*) Photomicrograph of kidney section of a 10-month-old mouse with severe parenchymal fibrosis.(*D*) Photomicrograph of a region of the same mouse kidney shown in (*C*), showing renal tubular hyperplasia. Magnification approximately $40 \times$. g, glomerulus; f, fibroblasts; t, tubule. (*E*) Transmission electron microscopy of the glomerular deposit of a $UG^{-/-}$ mouse with severe renal disease. Magnification approximately $6000 \times$. (*F*) The inset in (*E*) is magnified ($60,000 \times$), which shows the presence of long striated fibrillar structures consistent with the presence of collagen (*col*) and short diffuse ones consistent with Fn fibrils. (*G*) Fn immunofluorescence of a kidney section from a $UG^{+/+}$ mouse using murine Fn antibody. Note the absence of Fn-specific immunofluorescence in the glomeruli marked 'g' of a $UG^{+/+}$ mouse, (*H*) Fn immunofluorescence of a kidney section from a $UG^{-/-}$ mouse with severe renal disease. Note the intense Fn immunofluorescence over the glomeruli. Mason's trichrome staining of the kidney sections from $UG^{+/+}$ (*I*) and $UG^{-/-}$ (*J*) mice. Note the presence of bluish staining over the glomeruli of $UG^{-/-}$ mouse kidney section indicating the presence of collagen. Magnification approximately $40 \times$. (Reprinted with permission from: Zhang Z. et al. (1997) Science **276:** 1408–1412. Copyright © 1998, American Association for the Advancement of Science.)
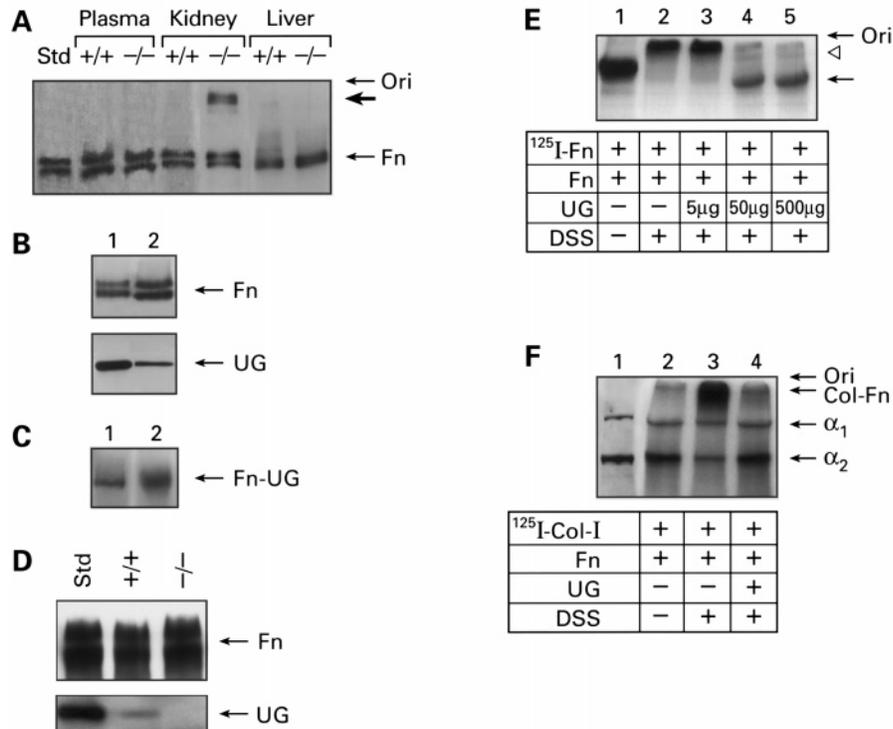
Figure 9. Detection of multimeric Fn in $UG^{-/-}$ mice and the effect of UG on Fn-Fn and Fn-collagen interactions. (*A*) Immunoprecipitation and Western blotting of Fn from plasma, kidney and liver of $UG^{+/+}$ and $UG^{-/-}$ mice. Immunoprecipitates were resolved on 4–20% (liver) and 6% (kidney and plasma) SDS-polyacrylamide gels, respectively, under reducing conditions. Bold arrow indicates the multimeric Fn band present only in the kidney lysate of $UG^{-/-}$ mice. (*B*) Binding of UG with Fn followed by coimmunoprecipitation and detection by Western blotting. The immuoprecipitated proteins were resolved by electrophoresis either on 6% or 4–20% gradient SDS-PAGE under reducing and denaturing conditions for Fn and UG, respectively. Note that the immunoprecipitates contain both Fn (lane 2, upper panel) and UG (lane 2, lower panel). Lane 1 of both panels represent corresponding standards stained with Coomassie blue. (*C*) The Fn-^{125}I-UG complex was immunoprecipitated with Fn antibody, and the immunoprecipitate was resolved by electrophoresis on 6% nonreducing, nondenaturing polyacrylamide gels. Lane 1 is the Coomassie blue-stained Fn-UG heteromer, and its autoradiogram is shown in lane 2. Note that there is no appreciable difference in the electrophoretic mobilities of the heteromer compared with Fn alone, as the slight increment in molecular mass of the UG-Fn heteromer cannot be discriminated from that of Fn under the experimental conditions used. (*D*) Coimmunoprecipitation of Fn and UG from the plasma of $UG^{+/+}$ and $UG^{-/-}$ mice. Fn (upper panel); UG (lower panel). Std, standards for UG and Fn. (*E*) Affinity cross-linking of ^{125}I-Fn with unlabeled Fn in the absence (lane 2) and presence of varying amounts of UG (lanes 3–5). The intensity of the very high molecular weight, radioactive Fn band (lane 2) formed in the absence of UG is reduced in a dose-dependent manner. Lane 1, ^{125}I-Fn with unlabeled Fn in the absence of UG and DSS. Open arrowhead, multimeric Fn; lower thin arrow, 220-kDa Fn. (*F*) Affinity cross-linking of ^{125}I-collagen-I with unlabeled Fn in the absence (lane 3) and presence (lane 4) of UG. Lane 1, Coomassie blue-stained collagen-I; α_1, α_1-chain of collagen-I; α_2, α_2-chain of collagen-I. Lane 2, ^{125}I-collagen-I and unlabeled Fn in the absence of UG and DSS. (Reprinted with permission from: Zhang Z. et al. (1997) Science **276:** 1408–1412. Copyright © 1998, American Association for the Advancement of Science.)

it has many and varied biological effects, not unlike cytokines and (iii) it has a receptor through which UG regulates cellular functions [145, 146]. Of course, these are only speculations at this time, and we realize that the biological properties of UG discussed above do not warrant calling this protein a cytokine. Nonetheless, many properties of this protein are reminiscent of those characteristic of cytokines.

To understand the phenotypic manifestations of UG deficiency, we have employed more sensitive techniques (e.g. competitive RT-PCR, using total RNA from isolated glomeruli and not from the whole kidney tissues of knockout and normal mice), and we were able to demonstrate that Fn-mRNA levels are significantly higher in isolated glomerular mesangial cells of $UG^{-/-}$ mice compared with those of its $UG^{+/+}$ counterpart (F.

Zheng et al., unpublished results). Thus, it appears that in UG-deficient mice, Fn production in the glomeruli may be dysregulated. Moreover, we also found that glomerular messangial cells from both UG$^{-/-}$ and UG$^{+/+}$ mice equally express the UG receptor (F. Zheng et al., unpublished results). In light of these results, it is entirely possible that disruption of the UG gene creates a deficiency of the ligand (i.e. UG), and as a result genes downstream from the UG receptor-mediated pathway may be dysregulated. Ongoing studies of the cloning and characterization of UG receptor cDNA and gene, and the mechanism(s) of signal transduction via this pathway are expected to broaden our knowledge of some of these aspects of UG function. Moreover, UG knockout mice provide a valuable animal model to explore the molecular mechanisms of human glomerulopathies in general and familial Fn-deposit glomerulopathy [178, 179] in particular. The fact that UG knockout mice with late onset disease develop both glomerulopathy and renal parenchymal fibrosis tempt us to speculate that UG may be one of the endogenous antiinflammatory/immunomodulatory factors, the physiological functions of which include the maintenance of normal glomerular function.

For more than 3 decades UG has been the subject of intense investigations. The results of these investigations have clearly established that this is a multifunctional protein that is physiologically important. The discovery of high-affinity UG-binding proteins [144–146] adds a new dimension to our understanding of the multifaceted nature of this protein. Future studies may elucidate whether UG and proteins having sequence similarities to it belong to a novel cytokine/chemokine family.

Note added in proof. While this manuscript was in press, we noted that Chilton and Hewetson have published a comprehensive review on the role novel elements that are important in UG/CC 10 kDa protein expression: Chilton B. S. and Hewetson A. (1998) Zinc finger proteins RUSH in where ohters fear to tread. Biol. Reprod. **58:** 285–294.

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