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Type 3 cystatins; fetuins, kininogen and histidine-rich glycoprotein

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1. ABSTRACT

This review describes the properties of four structurally related, abundant plasma proteins denoted fetuin-A/alpha-2-Heremans Schmid-glycoprotein (AHSG), fetuin-B (FETUB), kininogen (KNG) and histidine-rich glycoprotein (HRG). These proteins form a subgroup (denoted type 3) within the cystatin superfamily of cysteine protease inhibitors. Apart from KNG, the type 3 proteins appear to lack cystatin activity. AHSG has its major function in regulation of bone mineralization; the physiological role of FETUB is poorly understood. KNG serves dual functions in the assembly of the protein complex initiating the surface-activated blood coagulation cascade and as a precursor for the kinin hormones. The heparin-binding HRG has also been implicated in regulation of coagulation. In addition, several members of the type 3 cystatins have been implicated in tumor growth and shown to regulate endothelial cell function and formation of new blood vessels, angiogenesis. Thus, these proteins may potentially be useful in treatment of diseases characterized by excess angiogenesis such as cancer.

2. INTRODUCTION

The evolutionary conserved cystatins (inhibitors of cysteine proteases) were identified in the 1960s. Since then, the cystatin family has been divided into type 1 (mainly intracellular proteins), type 2 (mainly extracellular proteins), and type 3 cystatins (plasma proteins). The cystatins inhibit cysteine peptidases of the papain family and play key roles in a wide array of physiological processes as well as in disease. The type 3 family members fetuin-A/alpha-2-Heremans Schmid (HS)-glycoprotein (AHSG), fetuin-B (FETUB), kininogen (KNG) and histidine-rich glycoprotein (HRG) are disulfide-bonded, multi-domain proteins with more than one cystatin-like domain; AHSG, FETUB and HRG each contain two tandem cystatin domains, whereas KNG contains three cystatin domains. KNG displays cystatin-activity whereas the other type 3 family members appear not to be functional cystatins (1). On the contrary, AHSG has been identified as a potential positive regulator of the cysteine protease m-calpain in wounded cells (2). The type 3 members are glycoproteins produced mainly in the liver, which exist in

Table 1. Approved symbols for genes and proteins

Alpha-2-HS-glycoprotein/fetuin-A
1. Approved name: Alpha-2-HS-glycoprotein. Also denoted fetuin-A.
2. Approved symbol: AHSG
3. Accession no: P02765
Fetuin-B
1. Approved name: fetuin-B
2. Approved symbol: FETUB
3. Accession no: Q9UGM5
Kininogen
1. Approved name: kininogen
2. Approved symbol: KNG
3. Accession no: P01042
Histidine-rich glycoprotein
1. Approved name: histidine-rich glycoprotein
2. Approved symbol: HRG
3. Accession no: P04196

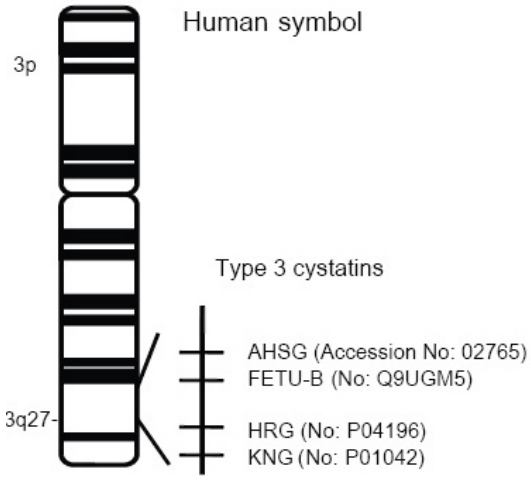

Figure 1. Organization of the type 3 cystatin family genes on human chromosome 3.

serum at high concentrations. AHSG has its major role in regulation of mineralization (3). FETUB has a similar tissue distribution as AHSG and the two proteins are coregulated, possibly suggesting overlapping functions (4). Both AHSG and FETUB have been implicated in regulation of tumor growth (5-7). KNG is produced as several splice variants, high and low molecular weight KNG (HK and LK respectively), from which the hormone bradykinin is generated. Bradykinin is a potent inflammatory mediator that causes vasodilation and enhanced capillary permeability (8). Gene inactivation of one of two murine KNG genes causes delayed injury-induced thrombosis (9). HRG is also important in regulation of clotting and fibrinolysis (10). In addition to their function in regulation of coagulation, KNG and HRG serve as regulators of angiogenesis.

3. NOMENCLATURE

Below follows the approved names, symbols and Uniprot accession numbers of the type 3 cystatin family members according to the HUGO Gene Nomenclature Committee (11); also, see http://www.genenames.org). For clarity and simplicity, we will use the approved symbols for genes as well as proteins in capitals irrespective of species throughout (if pertinent, the species will be indicated in the text)(Table 1).

4. CHROMOSOMAL LOCALIZATION AND GENE ORGANIZATION

The cystatin type 3 proteins are encoded by single-copy genes. The AHSG and FETUB genes are positioned immediately adjacent to the KNG and HRG genes on mouse chromosome 16 and human chromosome 3 (3q27) (Figure 1).

The human AHSG gene encompasses 7 exons spanning around 8.2 kb. Two common single nucleotide polymorphisms (SNPs) have been detected in the human; 6826C/T in exon 6 and 7495C/G in exon 7, which modulate circulating serum levels of AHSG (12).

The mouse FETUB gene consists of eight exons. The coding sequence starts in exon 2. Two alternative first exons encoding distinct 5'UTRs exist and have been denoted isoforms 1 and 3. Isoform 2 uses the same 5'UTR as isoform 1, but isoform 2 lacks exon 2 due to alternative splicing (13). These data have been derived from in silico cloning and await experimental confirmation. Thus, the physiological role of the putative splice variants is unclear.

There are two homologous mouse KNG genes (9) which are arranged head-to-head; there is only one KNG gene in the human. The human KNG comprises 11 exons encompassing about 27 kb. The two molecular weight variants of KNGs, low molecular weight kininogen, LK, and high molecular weight kininogen, HK, arise through alternative splicing.

The human HRG gene comprises 7 exons encompassing 12 kb. Notably, the largest exon VII encodes the entire C-terminal half of HRG. There are no HRG splice variants. Two different molecular weight forms, 75 and 77 kDa, of HRG in human blood samples have been described (14). The 77 kDa variant contains a serine residue at position 186, allowing attachment of an N-linked carbohydrate group. The 75 kDa form instead carries a proline at position 186. Three cases of congenital deficiency of HRG with familiar thrombophilia have been reported. All thrombophilic probands are women and their plasma HRG level is 20-50% of normal level. Two Japanese cases have been denoted Tokushima 1 and 2 (15, 16). The Tokushima 1 HRG variant contains a mutation resulting in exchange of Gly to Glu at position 85 in the first cystatin domain, which causes enhanced intracellular degradation of HRG. In the Tokushima 2 HRG, a mutation resulting in exchange of Cys to Arg at position 223 in the second cystatin domain has been identified.

5. STRUCTURE OF TYPE 3 CYSTATINS

The type 3 cystatins are multi-domain proteins containing aminoterminal cystatin domains (Figure 2). At

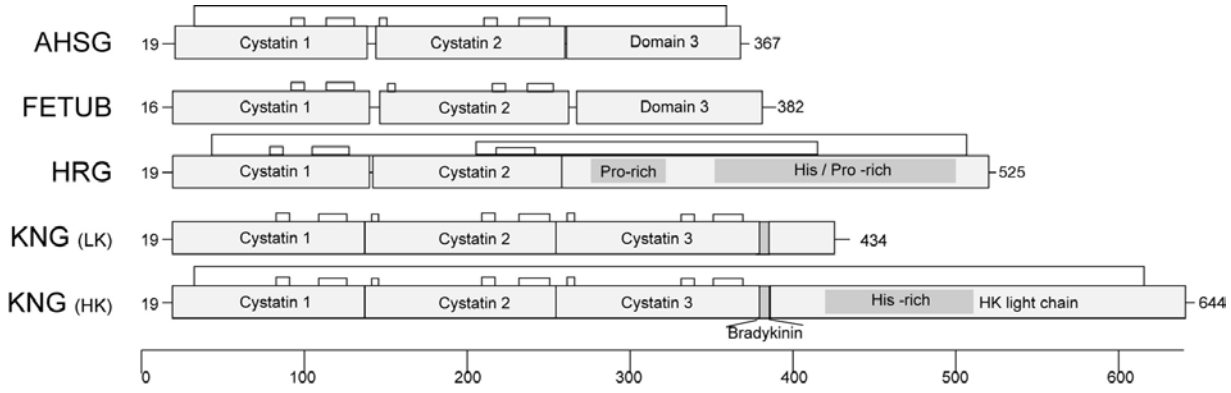

Figure 2. Schematic structures of type 3 cystatins. Domain organization is shown with indications of disulfide bridges, cystatin domains, and the His/Gly and His/Pro domains in KNG and HRG, respectively.

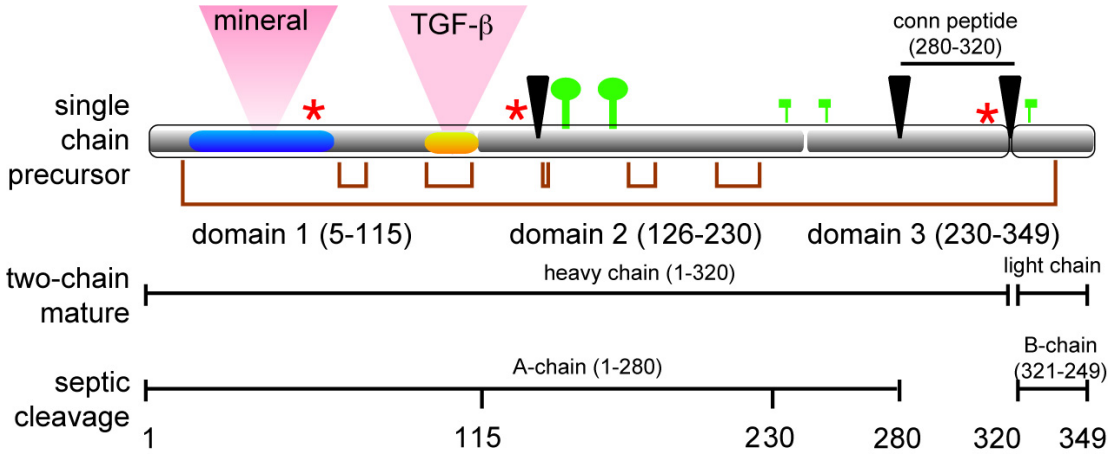

Figure 3. Post-translational modification of AHSG, a prototypic type 3 cystatin familiy member. AHSG consists of three domains, D1-D3, of ~115 amino acids each. Disulfide bridges indicated in brown rectangles are characteristic of the tandemly arranged cystatin domains D1 and D2. One interdomain disulfide links domain D3 to domain D1. Proteolytic cleavage sites are indicated by black wedges, serine phosphorylation sites are marked as red asterisks, N-glycosylation sites as large green and O-glycosylation sites as small green beacons. The so-called connecting peptide of AHSG is indicated. Binding regions for apatite and TGF-ß like growth factors are shown as pink triangles. Please note that carbohydrate binding proteins and phosphoamino acid-specific ligands may also bind and influence AHSG activity and stability.

this point, none of these proteins have been analyzed by x-ray crystallography, but computer modeling of the cystatin domains have been presented (17).

Post-translational modifications are common in the type 3 cystatins. Figure 3 illustrates post-translational modifications and binding motifs in AHSG, which is produced as a single chain precursor. The precursor is proteolytically processed to yield the mature circulating plasma form of AHSG comprising a heavy and a light chain (18). This first cleavage occurs by a secretory protein Golgi protease concomitantly with Ser/Thr phosphorylation (19) and addition of N-linked carbohydrate groups. Proteolysis in septicemia can cleave another 40 amino acids from the C-terminus of the heavy chain, forming the so-called A-chain (see Figure 3). The 40 amino acid residue C-terminal stretch has been termed "the connecting peptide" as it connected the combined A- and B-chains to the contiguous translated cDNA sequence when this became available. A substantial fraction of AHSG isolated from human plasma is serine phosphorylated (19); serine phosphorylation of AHSG has been implicated in its negative regulation of the insulin receptor tyrosine kinase (20) but is dispensable for the ability of AHSG to regulate mineralization (17). It is likely that these many post-translational modifications, which have been identified in human as well as bovine AHSG (21), may regulate activity, tissue targeting and plasma half-life.

HK and LK KNGs are organized in an identical heavy chain consisting of domains 1, 2 and 3 (see Figure 2). While HK contains a 56 kDa light chain encompassing domains 5 and 6, LK contains a unique light chain of 4 kDa. In both HK and LK, the light and heavy chains are linked by domain 4, which encodes the nonapeptide bradykinin (22). Domain 5 in HK contains repeats of histidine and glycine residues (denoted the His/Gly domain), which confer binding of metal ions such as Zn^{2+} (23).

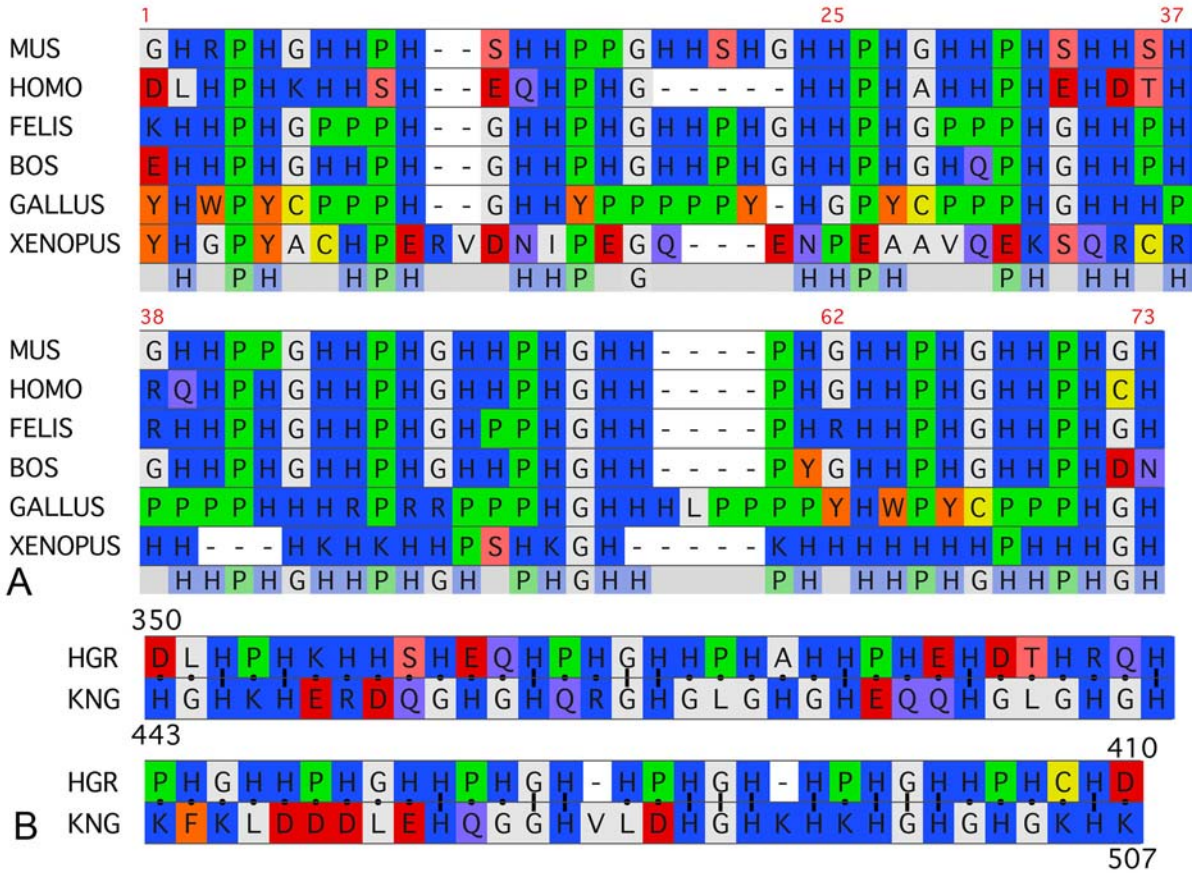

Figure 4. Structural alignments of the His-rich domains in cystatin type 3 family members. (A). Multiple protein-sequence alignment of HRG-His/Pro repeats in different species (mouse, man, cat, cow, chicken and frog). The alignment was done using the MUSCLE algorithm (66) and visualized in BioX. All sequences were extracted from the ENSEMBL, release 48 (67). Amino acid sequence positions for human HRG are indicated. (B). Pairwise alignment between the human repeat regions shows sequence similarity between the His/Pro domain of HRG and the His/Gly domain of KNG. The corresponding amino acid positions are shown for each protein sequence. Default parameters were sued for the alignment using the BioX software package (BioX; a graphical user interphase (GUI) to the eBiotools package. Erik Lagercrantz, Ville Jutvik, Alvaro Martinez Barrio and Erik Bongcam-Rudloff. Manuscript). Lines indicate identity, dots indicate non-identity.

The central part of HRG contains 12 consecutive repeats of 5 amino acid residues, dominated by histidine and proline residues, and with the consensus sequence HHPHG (denoted the His/Pro domain; Figure 2). This organization is strongly conserved and appears in a variety of species (Figure 4a**).** A high proline-content is associated with lack of ordered structure and proline residues are often actively involved in protein-protein interactions (24). Histidine residues in HRG bind metal ions with a strong preference for Zn^{2+} (25), which probably stabilizes the three-dimensional structure of this domain. There is significant similarity, about 30%, between the amino acid sequences in the His/Gly domain of KNG and the His/Pro domain of HRG (Figure 4b), possibly indicating shared function.

6. PRODUCTION, DISTRIBUTION AND PLASMA CONCENTRATION OF TYPE 3 CYSTATINS

The fetuins, AHSG and FETUB, are most highly expressed in the liver from zebrafish to mice and humans (Figure 5) but are also found in the kidney, the choroids plexus and in all major organs during fetal development (for a review, see (26)). The serum concentration of AHSG is in the range of 0.4-1 mg/ml. In conjunction with acute inflammation, the serum concentration drops and AHSG is therefore denoted a negative acute phase reactant (27). Unlike AHSG, FETUB is more abundantly expressed in human females than in males (4). Although expression of AHSG as well as FETUB normally is largely restricted to the liver, expression can be induced during tumor progression. Thus, the fetuins have been detected in a wide range of human solid tumor cell lines and in murine primary skin carcinomas (7). Moreover, the serum level of AHSG is upregulated in some glioma tumor patients (28).

The HK and LK KNG variants are present in serum at a concentration of 80 μg/ml (HK) and 60 μg/ml (LK), respectively (29). HK is expressed in the liver and kidney in the mouse whereas LK is detected in most mouse tissues. The two mouse KNG genes share 91% homology at the exon level but show a difference in tissue distribution;

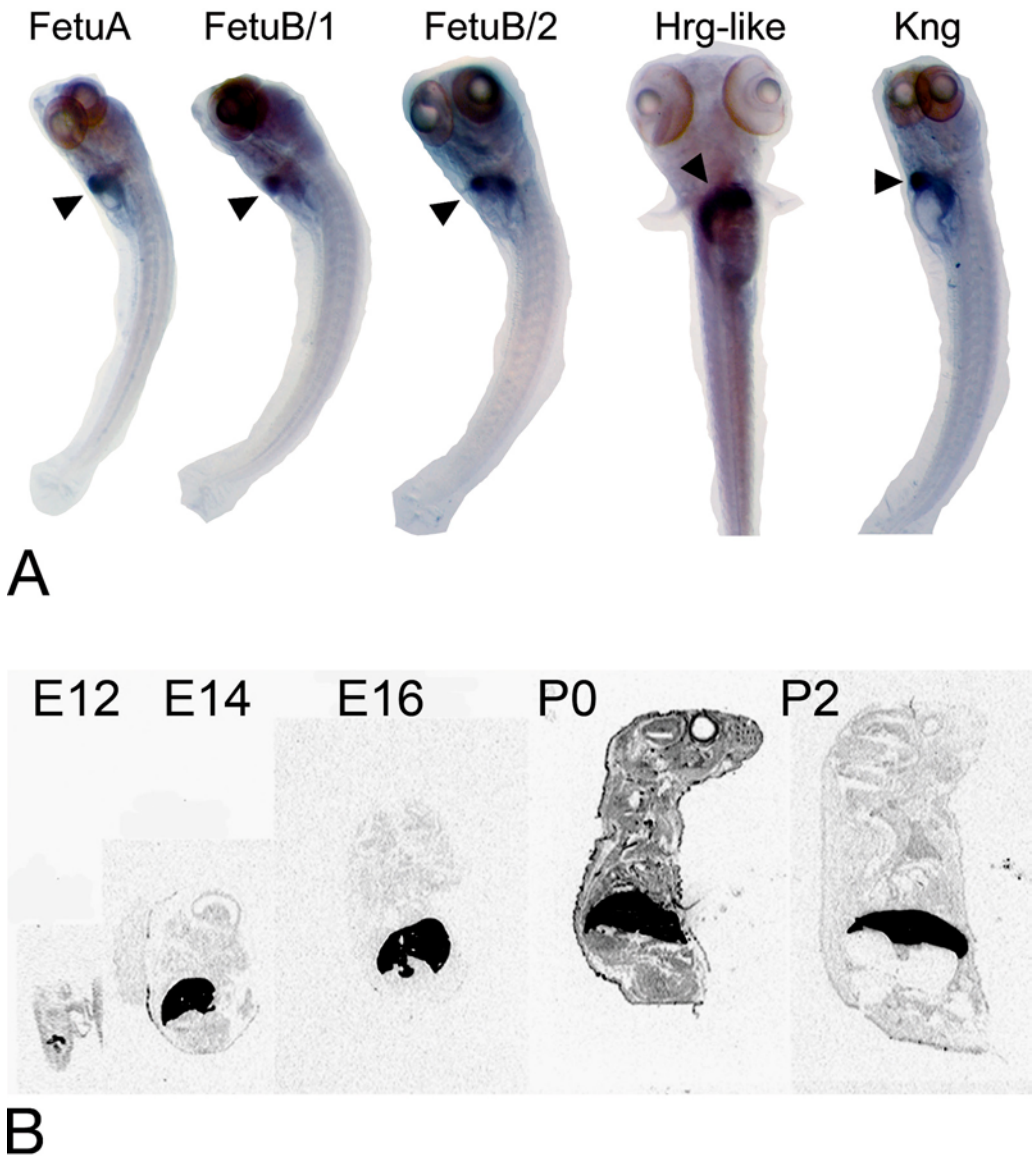

Figure 5. Expression pattern of type 3 cystatins. Expression of type 3 cystatin family members in (A) *Danio rerio* (zebrafish) at day 11 post fertilization (day 4 post-fertilization for HRG-like) and (B) AHSG expression in the mouse, judged from *in situ* hybridization of developing (E, embryonic day) embryos as well as post partum (P) animals. Note the dominating expression of AHSG in the liver. The zebrafish genome harbours seven type 3 cystatin genes. Panel A shows *in situ* hybridizations with probes synthesized to correspond to nucleotide sequences homologous to the indicated gene names. The arrowheads indicate expression in the liver. Please note however, that the "HRG-like" target sequence contains no HHPHG repeats and may therefore represent another fetuin-A/AHSG paralogue.

the mouse KNG1 HK transcript is expressed primarily in the liver and adrenal gland whereas the mouse KNG2 HK transcript is found in the kidney. Targeting of mouse KNG1 eliminates essentially all detectable HK and LK in plasma (9). The His/Gly repeat in HK binds Zn^{2+}, which is known to promote interactions with e.g. platelets and endothelial cells (30).

The concentration of HRG in serum is 100-150 μg/ml (31-33); it is present both as free protein and in α-granules of thrombocytes from which it becomes released in conjunction with thrombin activation (34). At birth, plasma levels of HRG are about 20% of those in the adult and levels increase further with increasing age (33). Furthermore, during pregnancy, maternal HRG levels undergo a marked reduction that progresses towards the third trimester (35). Plasma HRG concentration also decreases in patients suffering from advanced liver cirrhosis, AIDS, renal disease and pulmonary disorders such as asthma (31, 36, 37). Congenital disorders involving HRG mutations leading to changes in HRG expression levels and possibly function have been identified in a few

cases (15, 16). The central His/Pro domain of HRG (see Figure 2) is complexed with Zn^{2+}, which is required for many aspects of the reported physiological functions of HRG (see below). HRG is known to bind a variety of components in plasma with high affinity such as haem, plasminogen, fibrinogen, thrombospondin, immunoglobulin G, FcgR and complement (38); one very important binding partner of HRG is heparan sulfate which binds in a Zn^{2+}-dependent manner to the His/Pro repeats (39).

7. PHYSIOLOGICAL ROLES OF TYPE 3 CYSTATINS

7.1. Regulation of mineralization by AHSG

AHSG protects the body from unwanted mineralization, "calcification" in several ways:
(1) chemically, by binding to calcium phosphate nuclei and inhibiting further mineral growth (40), (2) biochemically, by stabilizing and opsonizing calciprotein particles (CPPs) to be cleared from circulation in time before the growing crystals reach a critical size at which they start to precipitate (17), (3) on the cellular level by alleviating the detrimental effects of calcium-overload during the shuffling of calcifying vesicles thus indirectly inhibiting apoptosis (41), (4) on a systemic level by binding and antagonizing transforming growth factor-β (TGF-β) and bone morphogenetic protein, thereby regulating their osteogenic activity (42, 43). Inhibition of formation of basic calcium phosphate by AHSG is dependent on one of its cystatin domains (17). Gene targeted (AHSG-/-) mice are phenotypically normal but develop severe calcification of various organs such as the kidney, the lung and the myocardium (44, 45) (see Table 2). This strongly suggests a role for AHSG as a "mineral chaperone" in mineral transport and clearing (46) and, more generally, a function for fetuin-like proteins as opsonins in phagocytosis and pathogen clearing and thus, in innate immunity.

7.2. Fetuins in regulation of tumor growth

AHSG has been implicated in regulation of signal transduction downstream of TGF-β and insulin, potentially resulting in impaired tumor growth and vascularization. Thus, AHSG has been shown to block TGF-β1 binding to its receptor, attenuating signal transduction and epithelial-mesenchymal transition. In sporadic human colorectal cancer specimens, the level of AHSG was three-fold lower than in normal tissue. The relevance of this finding was demonstrated by use of a mouse colorectal cancer model. Remarkably, the number and size of intestinal polyps was significantly increased in AHSG-/- mice compared to wild type mice (5). On the other hand, growth and dissemination of syngenic Lewis lung carcinoma was dramatically decreased in the AHSG-deficient mice (6). AHSG has also been implicated as an inhibitor of the insulin receptor tyrosine kinase (20, 47); the underlying molecular mechanism has not been established.

Hsu and colleagues identified the HRG and FETUB genes as linked to a susceptibility locus for skin tumor development on mouse chromosome 16 (7). They also showed that overexpression of FETUB in skin squamous carcinoma cells led to suppression of tumor growth in nude mice. FETUB-deficient mice are viable but the females are infertile in the homozygous knockout (Wesseling, Jahnen-Dechent *et al.* unpublished; see Table 2). It remains to be shown in the knock-out model whether FETUB indeed serves as a tumor suppressor.

It is unclear at this point if the different effects of AHSG on different mouse tumor models are relevant in a clinical scenario. Furthermore, it remains to be shown whether fetuins affect tumor growth by directly acting on tumor cells, or indirectly e.g. by modifying the extracellular matrix and thus regulating tumor infiltration of immune and endothelial cells.

7.3. Antibacterial function of KNG and HRG

Both the His/Pro domain in HRG (48) and the structurally related His/Gly domain in HK (49) exert anti-microbial effects by inducing breaks in bacterial membranes in a Zn^{2+}-dependent manner. The anti-bacterial activity is enhanced by low pH. A similar dependence on Zn^{2+} or low pH has been observed for various histidine-containing antimicrobial peptides. The antimicrobial effect could be due to binding of lipopolysaccharide (50), which is a major constituent of gram-negative cell walls. Whether HRG and D5 of KNG exert anti-bacterial effects *in vivo* remains to be settled.

7.4. Regulation of coagulation and platelet function by KNG and HRG

HK, along with prekallikrein and factor XII, is a component of the intrinsic activation system of coagulation, or the contact system (for a review, see (29)). HK inhibits thrombin-induced platelet aggregation and can also exert profibrinolytic activity (51). In agreement, targeting of one of the two mouse KNG genes, KNG1, leads to prolonged time to vessel occlusion in an arterial thrombosis model (9). However, proteins of the contact system seem to have little impact on hemostasis in humans. Thus, several examples of individuals with partial or complete loss of KNG expression due to autosomal mutations have been identified, who have had increased risk of thrombotic rather than hemorrhagic events (reviewed in (51)). Cleavage of KNGs by kallikrein results in release of the nonapeptide bradykinin (from HK) and lys-bradykinin (from LK). These kinins are associated with a wide range of functions but are best known for the ability to antagonize angiotensin-induced vaso-constriction and sodium retention (52).

HRG has also been implicated in hemostasis and families with reduced levels of HRG in plasma due to autosomal mutations have a higher incidence of thrombotic events (i.e. the Tokushima 1 and 2 cases (see section 4. ”Chromosomal localization and gene organization”). Inactivation of the HRG gene in mice is compatible with embryonic survival and presents no dramatic phenotype. There is however enhanced blood coagulation and fibrinolysis in HRGP-deficient mice, possibly due the loss of HRG as binding partner for fibrinogen as well as thrombospondin (10).

Table 2. Phenotypes of type 3 cystatin knockout mice

Name	Phenotype	Reference
Fetuin-A (AHSG)	"Mineral chaperone" soft tissue calcification, defective inhibition of apatite formation in serum	44, 45
Fetuin-B (FETUB)	Female infertility	Wessling, Jahnen-Dechent *et al.*, unpublished
Kininogen-1 (mKNG1)	Normal bleeding times but delayed arterial occlusion in thrombosis	9
Histidine-rich Glycoprotein (HRG)	Enhanced blood coagulation and fibrinolytic activities	10

7.5. KNG and HRG in regulation of angiogenesis

HK is cleaved by kallikrein to generate bradykinin and a kinin-free derivative denoted HKa. HKa, through the His/Gly domain in D5 (see Figure 2), induces apoptosis selectively of endothelial cells, in a Zn2+-dependent manner (53). The molecular mechanism of the anti-angiogenic effect of D5, which has been denoted kininostatin, involves decreased endothelial cell proliferation and migration dependent on modulation of the phosphatidyl inositol 3-kinase/Akt pathway (54, 55). D5 has also been shown to induce apoptosis through generation of intracellular reactive oxygen species (56). Furthermore, Wu and colleagues suggested that D5 binds to caveolin-enriched lipid membrane rafts, thereby inhibiting activation of αvβ3 integrin-dependent cytoskeletal reorganization and Rac1 activation in endothelial cells (57). Tropomyosin has been implicated as a transducer of the anti-angiogenic effect of HKa (58). The potential consequence of KNG-targeting (see Table 2) on physiological or pathological angiogenesis has not yet been reported.

HRG purified from rabbit was first identified in 2002 as an inhibitor of angiogenesis (59). Relatively high concentrations of HRG (100-500 nM) inhibited endothelial cell function in a range of in vitro assays. Angiogenesis in vivo in the chicken chorioallantoic membrane assay and in subcutaneous matrigel plugs was also affected by HRG. Olsson et al. validated these data using recombinant human HRG administered by subcutaneous injection, which potently inhibited vascularization of T241 fibrosarcoma in C57BL/6 mice (60). Inhibition of endothelial cell chemotaxis was used as an in vitro surrogate assay to show the potency of truncated versions of HRG added at a concentration of 1 nM. These data strongly indicated that the anti-angiogenic effect is mediated via the His/Pro domain in HRG (amino acid residues 330-389), and moreover, that this domain has to be released from the core protein in order to inhibit chemotaxis of endothelial cells. Subsequent work confirmed that the minimal active domain of HRG is located within the first 35 amino acid residues (aa 330-365) of the Zn2+-binding His/Pro repeat (61). The effect of HRG on endothelial cells was shown to involve the vitronectin-binding integrin αvβ3 causing uncoordinated activation of focal adhesion kinase (see Figure 6 for a schematic outline of the mechanism of action of HRG). In agreement, the ability of endothelial cells to form tubular structures in 3D collagen gel was disturbed (62).

Donate *et al.* (63) treated mice with 50-75 mg/kg/day of tetramers of the consensus sequence HHPHG, which caused decreased hemoglobin content in subcutaneous matrigel plugs, indicative of reduced angiogenesis. The treatment also caused delayed growth of xeno-transplants (3LL murine lung carcinoma and B16F1 murine melanoma). HRG, as well as the HHPHG repeat was shown to bind to the actin-binding protein tropomyosin in a Zn^{2+} and pH-dependent manner (63, 64) and tropomyosin was coined as the receptor for the anti-angiogenic activity of HRG.

HRG also has been implicated in stimulation of angiogenesis. Simantov and colleagues showed that HRG inhibits the binding of thrombospondin to its receptor CD36, thereby neutralizing the anti-angiogenic effects of thrombospondin (65). Thrombospondin binds to the aminoterminal part of HRG and therefore the ability of the released His/Pro-rich domain of HRG to exert its anti-angiogenic effect, is not influenced by thrombospondin. Clearly the net angiogenic effect in a physiological setting would be dependent on the balance of stimulatory and inhibitory factors. Growing evidence suggest that HRG contributes to this balance.

8. CONCLUSIONS

The type 3 cystatins are abundant plasma proteins whose physiological functions have been unclear. Thus, not until the availability of the knock-out model has the role of AHSG in mineralization been demonstrated (Table 2). The type 3 cystatins are not required for embryonic development as judged from the fact that targeting of each single type 3 cystatin family gene is compatible with survival of the embryo well into adulthood. The possibility that remaining cystatin 3 family members would compensate for the eliminated one has not been scrutinized but does not seem very likely. The highest degree of similarity in primary structure between the type 3 cystatins is within the cystatin domains. There is no consistent role of the cystatin domains in the type 3 proteins: Only the KNG cystatin domains possess actual cystatin activity. In AHSG, the cystatin domains do not exert cysteine protease inhibitory effects, but one of the domains is implicated in bone mineralization. In HRG, the cystatin domains need to be cleaved away to allow the His/Pro-domain to regulate endothelial cell function. Thus, the primary structure of the type 3 family members has this far not helped to resolve the biological functions of these proteins. More information on the three-dimensional structure of these proteins is warranted.

It is noteworthy that all cystatin type 3 family members have been implicated directly or indirectly in tumor growth. The mechanisms remain to be deduced,

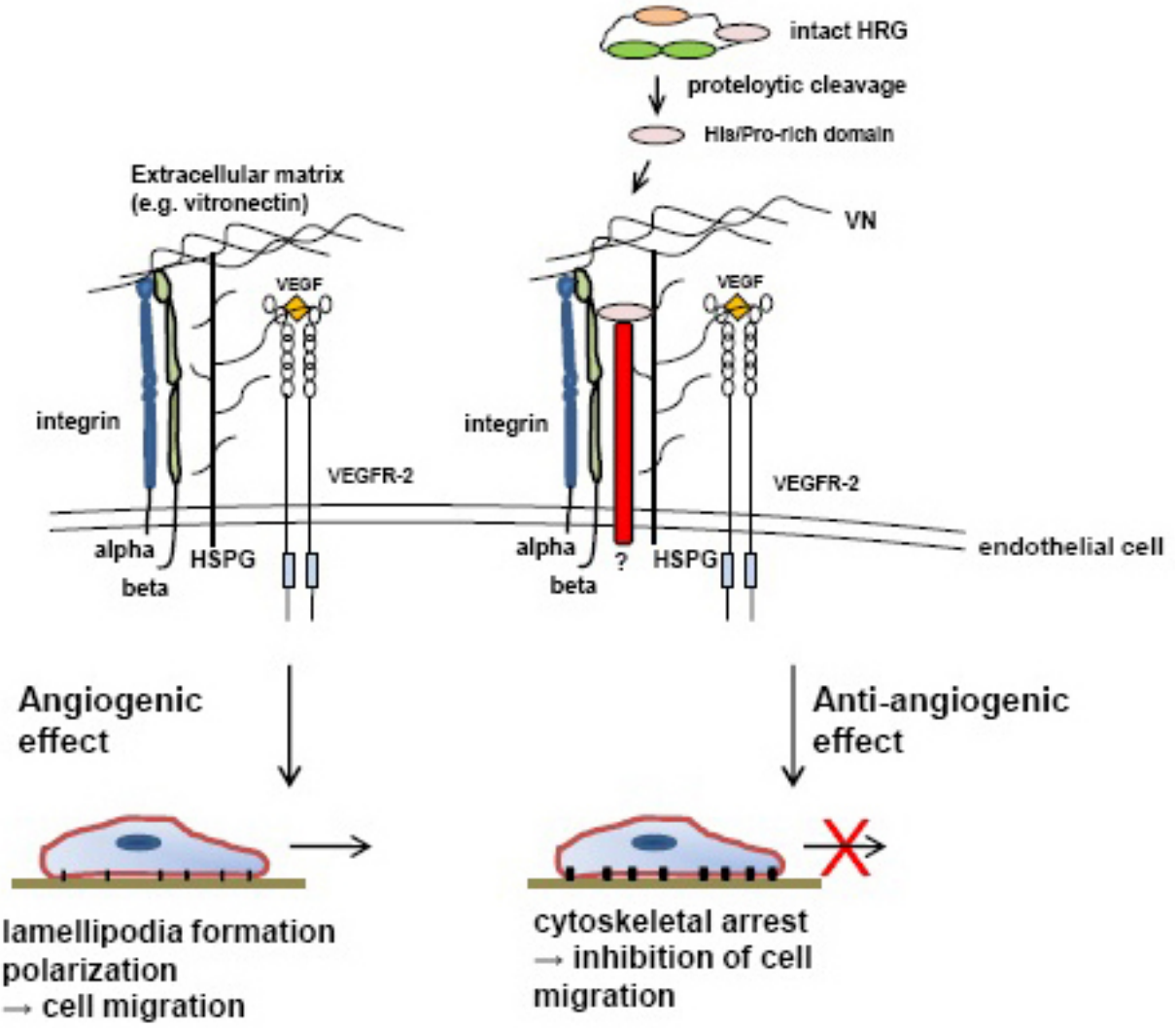

Figure 6. Schematic model of HRG effects on endothelial cell. The model has been adopted from ref. (61). HRG binds to its putative receptor (indicated in red), and in addition to one or several binding proteins (indicated here as integrin avb3 and heparan sulfate proteoglycans; HSPG). In the absence of HRG, vascular endothelial growth factor (VEGF) activation of VEGF receptor-2 leads to cytoskeletal rearrangement in endothelial cells, promoting endothelial cell migration and angiogenesis. In the presence of HRG, cytoskeletal rearrangements are disturbed and endothelial cell migration is inhibited, with consequent angiogenic arrest.

however. To ultimately understand how domain 5 (the His/Gly domain) of KNG and the His/Pro domain of HRG regulate angiogenesis, their receptors or binding proteins have to be identified. HRG binds heparin/heparan sulfate in a Zn^{2+}-dependent manner, through histidine residues in the His/Pro repeat (39). It is possible that HS serves as a critical binding protein for HRG in inhibition of endothelial cell function. Whether or not integrin $\alpha v\beta 3$ (61) or tropomyosin (58, 64) directly participate to transduce the effects of HRG and D5 on endothelial cells awaits further confirmation. Furthermore, more stringent analyses will have to be done of the vessel phenotype in tumor challenged, knockout mouse models, which are now available for all the cystatin type 3 family members.

9. ACKNOWLEDGEMENTS

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