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Smithgall T.E., The c-fes family of protein-tyrosine kinases, *Crit. Rev. Oncogenesis* **9**, 43–62.

Sawyers C.L., Chronic myeloid leukemia, *N. Engl. J. Med.* **340**, 1330–1340 (1999).

Smithgall T.E., Signal transduction pathways regulating hematopoietic differentiation, *Pharmacol. Rev.* **50**, 1–19 (1998).

Smithgall T.E. et al., Control of myeloid differentiation and survival by stats, *Oncogene* **19**, 2612–2618 (2000).

FETUIN/α2-HS GLYCOPROTEIN

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Fetuins/α2-HS glycoproteins are generic plasma-binding proteins in mammals. Fetuins ligands include fatty acids, minerals, peptide hormones, and cytokines. Fetuins have thus been shown to be involved in nutrition (1), immune response, bone formation and resorption, and hormone signaling. Illnesses possibly associated with fetuin include toxic shock, depressed cellular immunity, non-insulin-dependent diabetes, hepatitis, osteoporosis, and nephrocalcinosis. A growing body of structural information and whole animal studies of fetuin-null mutant mice finally enable us to critically evaluate these postulates.

Fetuin homologs occur in reptiles, birds, and marsupials. Bovine fetuin (derived from the latin word *fetus*) was first described in 1944 by Pedersen as the most abundant globular plasma protein in fetal calf serum. The human species homolog was independently identified by Heremans and Schmidt and Bürgi. It was later named α2-HS-glycoprotein by Schultze (2) in honor of two of the three original codiscoverers. The name also indicates that α2-HS glycoprotein comigrates with the α2-globulin fraction of serum proteins in cellulose acetate electrophoresis. This protein was repeatedly confused with α2-Z-glycoprotein/Zn α2 glycoprotein (Z for zinc-binding) or α-fetoprotein (AFP), two unrelated entities.

The genetic symbol for α2-HS glycoprotein is *AHSG*. It is cross-referenced in all major biological databases including GenBank, EMBL, SwissProt, OMIM, and MLC. Alternatively, A2HS, AHS, and HSGA have all been used. The protein database entries in SwissProt are A2HS_HUMAN, A2HS_MOUSE, A2HS_RAT and so on. The names "fetuin" and "α2-HS glycoprotein" are perfectly interchangeable. Although α2-HS glycoprotein mainly denotes the human protein, "fetuin" is used generic for this family of proteins. "Fetuin" reflects the fact that maximum serum levels of the protein are attained during the fetal period. The species homolog in rat was independently called "fetuin," "pp63" for phosphoprotein 63 kDa, and "BSP59" for bone sialoprotein 59 KDa. "Countertrypin," a trypsin inhibitor from mouse and Mongolian gerbil serum is identical to fetuin. Partial sequence of hemonectin, a cell adhesion molecule from rabbit bone marrow and blood were similar to fetuin. However, it is still controversial whether these proteins are identical (3). A fetuin-like structure is found in the snake antihemorrhagic factor (HSF), a metalloproteinase inhibitor, which prevents hemorrhagic and proteolytic activities of the Habu snake venom.

For a detailed introduction into the field the reader is referred to an excellent review of fetuins (4), summarizing the knowledge of fetuins accumulated before 1995. In this contribution knowledge on fetuins accrued since, will be mainly discussed.

GENE STRUCTURE AND GENETIC POLYMORPHISM

Fetuins are single-copy genes, but closely related proteins were recently added to public databases (fetuin-related protein, fetuin B, accession AJ242926). The protein structure and physiological role of this latter form of fetuin is presently unknown. The genomic organization of human α2-HS glycoprotein/fetuin (5), rat fetuin, and mouse fetuin genes (6) are published. The structures of fetuin genes reflect the domain structure of fetuin proteins. Each fetuin gene consists of seven exons and six introns. Beginning at the 5′-end of the gene a tandem arrangement of three short exons each (84–280 bp) encode two cystatin-like domains D1 and D2. Fetuins are thus members of the cystatin superfamily of proteins. Further members of this superfamily sharing cystatin-like domains are Histidine-rich glycoproteins (HRG) and Kininogens (KNG). The cystatin domains originated from gene duplication, as AHSG, HRG, and KNG map within 1 Megabase of DNA on chromosome 3q27 in humans. Exon 7 of fetuin (>400 bp) encodes domain D3 of fetuin. This exon diverges between fetuins and bears no obvious structural similarity with other known proteins.

Several codominant alleles of fetuin cause polymorphism in human populations. Two common alleles give rise to three major phenotypes characterized on the genome level (7). The threonine(Thr) residues Thr230 and Thr238 in the allele AHSG*1 are exchanged for methionine(Met) and serine(Ser), respectively in AHSG*2. Phenotypically, AHSG polymorphism presents itself as charge polymorphism on either IEF gels or 2D gels. Since Thr, Ser, and Met residues do not change protein net charge, the Thr and Ser residues must be differentially glycosylated and or phosphorylated in the allelic variants. In fact, Thr238 in AHSG*1 is O-glycosylated. Altogether over 15 phenotypic variants of fetuin have been described. Owing to this polymorphism α2-HS has been extensively used as a forensic marker. Despite the widespread screening of α2-HS polymorphism complete deficiencies have never been reported indicating a vital role of α2-HS glycoprotein or fetuin.

REGULATION OF GENE EXPRESSION

Fetuins are plasma proteins predominantly made in the liver. Northern hybridization revealed that at any given time during fetal development, the fetuin RNA production in liver will contribute over 95% of the total fetuin gene expression in fetal rats. In situ hybridization and molecular cloning of EST's show however, that fetuin mRNA is expressed in all major organ systems and furthermore that during fetal development local expression can be quite high at the level of individual cells. For example, cells of the choroid plexus of developing sheep brain, cells of the hematopoietic system (8), and cells of the developing major organ systems in kidney, gut, and skin in fetal rats all transiently produce high amounts of fetuin mRNA (9), as indicated by strong local staining during in situ hybridization.

The strong and live-long liver-derived expression of fetuins can be explained on the gene level. Fetuin gene promoters contain C/EBP and HNF-1 binding sites, which are responsible for strong gene expression in liver tissue. Promoter analysis having chloramphenicol acetyl transferase (CAT) reporter constructs demonstrated that the rat fetuin gene promoter is one of the strongest basal promoters known, comparable to viral promoters and stronger than the albumin promoter. As a consequence, fetuin mRNA is highly abundant during periods of maximum gene expression. The overall peak period of expression, which is mainly liver-derived, varies between species. It is best generalized as prenatal or embryonic in large animal species and postnatal in small animals. This timing roughly coincides with the onset of skeletal mineralization at the scale of the entire organism, indicating a role of fetuins in the regulation of mineral deposition. On the level of individual organs however, fetuin mRNA expression peaks during the switch from cell proliferation to differentiation, suggesting a role for fetuins in cellular growth control. Specific elements to regulate this extrahepatic fetuin expression have yet to be described.

Fetuin is considered a negative acute phase protein in humans and rats. Whenever studied at the transcriptional level, fetuin genes were transcriptionally downregulated during sepsis and trauma. Cytokine response elements mediating this negative regulation have been identified in the human, rat, and mouse fetuin gene promoter (10,11). Bovine fetuin might, however, react as a positive acute phase protein after trauma due to mobilization of fetuin protein from high-capacity stores like bone tissue.

FETUIN PROTEIN STRUCTURE

Fetuins are soluble, acidic, globular, sialoglycoproteins. The calculated molecular weight of mature α2-HS glycoprotein/human fetuin (M 37,441) is much lower than the apparent molecular weight of 50 to 60 kDa observed in denaturing SDS-gel electrophoresis. This discrepancy reflects the overall acidic nature of fetuins (IP 4.1–4.7) and extensive posttranslational modifications. A cartoon of human α2-HS is given in Figure 1. Individual domain structures, ligand-binding regions, disulfide structure and posttranslational modification sites are indicated.

Fetuins are members of a structurally well-preserved family of glycoproteins within the cystatin superfamily. This

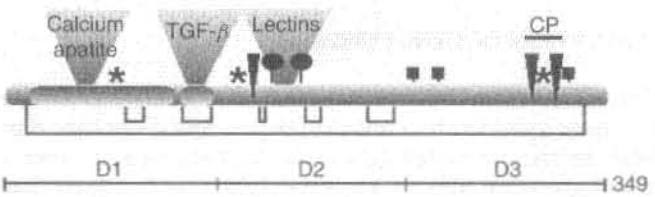

Figure 1. Structural organization of α2-HS glycoproteins / fetuins. Fetuins consist of three domains D1-D3 of approximately 120 amino acids each. Disulfide bridges indicated by lines are characteristic of the tandemly arranged cystatin domains D1 and D2. One interdomain disulfide links domain D3 to domain D1. Proteolytic cleavage sites are indicated by arrow heads, serine phosphorylation sites are given as asterisks, N-glycosylation sites as large and O-glycosylation sites as small beacons. The so-called connecting peptide of human α2-HS is marked "CP." Binding regions for apatite, TGF-β like growth factors and lectins are given as triangles.

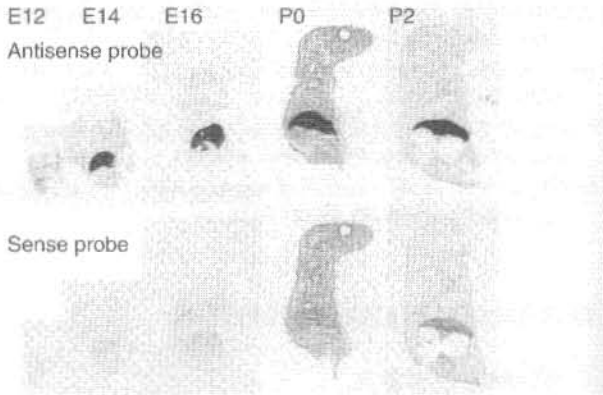

Figure 2. Fetal and perinatal expression of mouse fetuin RNA. Longitudinal mouse tissue sections were hybridized with 35S-labelled antisense (top row) or sense (bottom row) fetuin cRNA. Probe hybridization was visualized by sheet film autoradiography. Liver anlage (E12) or liver tissue invariably yielded the highest hybridization signals.

relationship was discovered by the similarity-searching of a protein sequence database and confirmed, when the disulfide bond structure of α2-HS was determined. Five disulfide bonds are linearly arranged in two tandem cystatin repeats (Fig. 1), forming domains D1 and D2. A sixth disulfide bond attaches the amino-terminal end of the protein to the carboxy-terminus. Twelve cysteine residues are thus conserved in all fetuin sequences, suggesting a common disulfide structure. This structure was termed fetuin family signature 1 in the database Prosite *(http://www.expasy.ch/cgi-bin/nicedoc.pl?PDOC00966)*. The carboxy-terminal domain D3 diverges between fetuins and is not present in other known proteins. Domain D3 is however, rich in glycine and proline and is therefore referred to as "collagen-like." A second feature characteristic of fetuins called fetuin family signature 2 comprises the sequence motif L-E-T-x-C-H-x-L-D-P-T-P. This sequence contains the first conserved cysteine of disulfide loop 1 in domain D1 (Fig. 2). This sequence is conserved in fetuins A, but is truncated (L-E-T-x-C-H-x-L) in human and rodent fetuins B.

POSTTRANSLATIONAL MODIFICATION

Fetuins are glycoproteins having both N and O-linked sugar moieties accounting for roughly one-tenth (α2-HS) to one-fourth (bovine fetuin) of the molecular weights. Fetuin glycan chains are sialylated and partly sulfated. Due to this complex glycosylation pattern, fetuins serve as model substances for glycoprotein chemistry and hemagglutinin / lectin research. On a practical note, the strong binding of pertussis toxin to the terminal sialic acid residues in fetuin form the basis of an FDA-approved pertussis toxin test. Furthermore, α2-HS glycoprotein or fetuin-binding and sequestration of lectins proved a major complication in experimental cytotoxic therapy using cancer cell specific antibodies coupled to the (*Rizinus communis*) agglutinin, ricin. Lectin-binding should always be regarded a possibility when fetuin binding to cells and to extracellular matrix is considered. Hepatic uptake of fetuin is mediated by the asialoglycoprotein receptor. Owing to its wide range of glycosylation variants fetuins can interfere with the uptake and distribution of sialylated

and N-acetylgalactosamine (GalNAc)–sulfated glycopeptide hormones like thyrotropin (12).

Fetuins undergo complex posttranslational modification. In hepatoma cells and in rat hepatocytes, fetuins are constitutively secreted as serine phosphorylated single-chain phosphoproteins. Phosphorylation is critically required for some fetuin functions but seems to be dispensable for others. Biological activity of fetuins has long been suspected to depend on modification by limited proteolysis. In particular, α2-HS glycoprotein/human fetuin naturally circulates as a heterodimer of a heavy chain and a light chain, which are derived by proteolytic processing of a common precursor. Further proteolytic degradation of human fetuin can liberate the so-called "connecting peptide," forty amino acids bridging the gap between the combined chains A and B and the contiguous cDNA sequence. The liberation of connecting peptide is effectively prevented when serum is drawn in the presence of protease inhibitors. Proteolytic processing does however, proceed in septicemic patients or in bulk protein preparations contaminated with trace amounts of chymotryptic activity (13). With the exception of a dibasic proteolytic cleavage sites at the border of domain D1 to D2 (Fig. 1), the proteolytic cleavage sites required for α2-HS glycoprotein/human fetuin processing are absent in all other fetuins. Therefore, the liberation of connecting peptide and subsequent proteolysis is likely to be occurring in human fetuin only. Any biological activity associated with this peptide would also be limited only to humans.

LOCALIZATION OF FETUIN PROTEIN EXPRESSION

Fetuin is a major plasma protein having a mean plasma concentration of 0.63 ± 0.2 g/L (~10 μmol/L) in adult humans. Fetuin can potentially reach all extracellular spaces in the body, travelling along the blood stream. Accordingly, fetuin is detected ubiquitously, using immunohistochemistry (IHC). Detecting fetuin protein using antibodies however, requires great care and caution. First, fetuins are generally highly immunogenic. Therefore, several reports in the literature where fetuin was detected in exotic places like erythrocytes or retina cells probably reflect the fact that traces of fetuin copurified with proteins used for immunization, and hence the antibodies reacted with more than one protein. Second, fetuin antisera elicited against pure preparations of fetuins from even closely related species hardly cross-react. Therefore, antibody assays or antibody staining are only meaningful when species-specific matching antibodies are used. Nevertheless, fetuin protein is present throughout the body. The protein half-life is 1.4 days (determined in rabbits) and the proteins gets selectively trapped in the mineralized bone and teeth tissue as a major noncollagen protein. Protein-clearing occurs through liver, kidney, and gut.

An expression survey in mice and in rats showed that the liver produces by far, the most fetuin at any time throughout fetal and postnatal life. Fetuin expression can be detected as early as E12 in fetal mice when mesenchymal cells form the liver anlage. Figure 2 shows that the liver constitutively produces fetuin at a rate of at least one order of magnitude higher than any other organ (14,15). The bulk of fetuin protein circulates in the blood and is eventually sequestered in the mineralized tissues of bone and teeth. In these tissues fetuin is selectively enriched over other plasma proteins to levels of several milligrams per gram bone. It must therefore, be regarded as a major noncollagen bone protein. This tissue distribution suggests a role for fetuin in bone mineral formation and turnover. Besides liver, fetuin is, however, also expressed transiently at high levels in all major organ systems (8,9). Peak expression occurs in structures undergoing differentiation and transformation around the time period in which cellular phenotypes are established. This expression pattern indicates a role of fetuin protein in cell differentiation and tissue formation.

BIOLOGICAL ROLE OF FETUINS

Many biological functions have been attributed to fetuins based on phylogenetic similarity, tissue distribution, regulation during disease, effects on cultured cells, and on biochemical grounds. Functions proposed are based on the fetuin's ability to influence processes as diverse as opsonization, lipid transport, cell proliferation, tyrosine kinase inhibition of the insulin receptor, protease inhibition, and hematopoietic cell-homing, all reviewed in an excellent monograph on fetuins (4). Recently, fetuin has been shown also to modify transforming growth factor-β(TGF-β) and bone morphogenetic protein (BMP) binding (16), hepatocyte growth factor (HGF) binding (17). Furthermore, several independent groups have shown in animal studies that fetuin might also interfere with lipopolysaccharide (LPS) and concomitantly with tumor necrosis factor (TNF-α) signaling (18–20). Confusing, but not necessarily contradictory, these results indicate molecular binding of fetuins to many diverse target molecules. Binding and sequestration of otherwise insoluble or potentially harmful ligands seems to be a salient feature of this and other major circulating plasma proteins like serum albumin, α2-macroglobulin, apolipoproteins, and so on, collectively referred to as "protective colloids" in historic literature on the role of plasma proteins. Besides the biochemical, cell culture, and localization studies, more whole animal studies are needed to clarify the role of fetuins. To this end, the generation of fetuin-deficient mice is a major advance. Genetic ablation of the gene for mouse fetuin has demonstrated that none of the proposed roles are indispensable during fetal or adult life. Fetuin-deficient mice are viable and fertile and show no gross anatomical abnormalities (6). Female exbreeder homozygous null mice develop mild ectopic calcification of soft tissues. Lethal ectopic calcification in these mice readily occurs when they are made hypercalcemic. Generally, the lack of a phenotype in unchallenged null mutant mice indicates genetic redundancy and may be considered evidence in favor of backup systems of critically important traits. Because the lack of fetuin does not seem to cause major defects under physiological conditions, the next logical step is to study fetuin-null mutant mice during pathophysiological challenges like acute phase, hormone challenge, infection, or induced hypercalcemia.

BIBLIOGRAPHY

1. Z. Nie, *Am. J. Physiol.* **263**, C551–C562 (1992).
2. K. Schultze and H. Haupt, *Naturwiss.* **49**, 15–17 (1962).
3. C. Peters et al., *Am. J. Med. Sci.* **309**, 285–294 (1995).
4. K.M. Dziegielewska and W.M. Brown, *Fetuin*, Springer-Verlag, Heidelberg, Germany, 1995.

5. M. Osawa et al., *Gene* **196**, 121–125 (1997).
6. W. Jahnen-Dechent et al., *J. Biol. Chem.* **272**, 31496–31503 (1997).
7. M. Osawa et al., *Hum. Genet.* **99**, 18–21 (1997).
8. K.M. Dziegielewska et al., *Histochem. Cell Biol.* **106**, 319–330 (1996).
9. O.B. Terkelsen et al., *Anat. Embryol. (Berl).* **197**, 125–133 (1998).
10. F. Banine et al., *Biochim. Biophys. Acta* **1398**, 1–8 (1998).
11. F. Banine et al., *Eur. J. Biochem.* **267**(4), 1214–1222 (2000).
12. M.W. Szkudlinski et al., *Endocrinology* **136**, 3325–3330 (1995).
13. P. Nawratil et al., *J. Biol. Chem.* **271**, 31735–31741 (1996).
14. T. Schinke et al., *J. Biol. Chem.* **271**, 20789–20796 (1996).
15. J.A. Kazi et al., *J. Biochem. Tokyo.* **124**, 179–186 (1998).
16. C. Binkert et al., *J. Biol. Chem.* **274**(40), 28514–28520 (1999).
17. T. Ohnishi et al., *Eur. J. Biochem.* **243**, 753–761 (1997).
18. O.Y. Chertov et al., *Immunol. Lett.* **42**, 97–100 (1994).
19. H. Wang et al., *Lancet* **350**, 861–862 (1997).
20. K.M. Dziegielewska and N.A. Andersen, *Biol. Neonate* **74**, 372–375 (1998).

FHIT

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Inactivation of the *FHIT* gene, encoded at 3p14.2, is one of the earliest and most frequent genetic changes in the development of human cancers. Reexpression of Fhit protein in cancer cell lines with *FHIT* deletions suppresses tumor formation by inducing apoptosis. The 1.5 Mb *FHIT* gene spans the FRA3B fragile site and is prone to deletions, accounting for frequent losses in carcinogen-exposed tissues. Fhit protein and its homologs in animals and fungi form a branch of the histidine triad superfamily of nucleotide binding proteins whose members possess diadenosine polyphosphate (Ap_nA) binding and hydrolysis activity. Fhit dimers bind two Ap_nA substrates in a manner that fills a deep, positively charged groove with substrate phosphates. Available evidence suggests that Ap_nA is a cofactor for the proapoptotic tumor-suppressing function of Fhit.

THE *FHIT* GENE IN NORMAL AND CANCER CELLS

Clear cell renal carcinoma, normally a disease of the elderly, occurs in young adults among carriers of a chromosomal translocation between the short arm of chromosome 3 and the long arm of chromosome 8. The t(3;8)(p14.2;q24) translocation occurs between the third and fourth exons of the *FHIT* gene (1). The FRA3B fragile site, as located by aphidicolin-induced breaks, is located within a 300-kbp region that begins 5′ of exon four and extends through exon five. In cancer cells, the *FHIT* gene frequently contains internal deletions that have removed coding exons and have been repaired by recombination between intronic LINE1 elements (2). In some cases, each *FHIT* allele has lost different exons and the common region of loss is within an intron. In cervical cancer, the fragile region of the *FHIT* gene is targeted by papilloma virus insertion (3). The primary manner by which *FHIT* is inactivated, that is, by deletions rather than point mutations, is similar to that of tumor-suppressor genes such as *CDKN2A* and is dissimilar to that of genes such as *TP53* that are frequent targets of missense mutations. Because *FHIT* has a 441 nucleotide coding sequence within a 1,500,000 nucleotide gene spanning FRA3B, selective pressure for defects in apoptosis is more often resolved by deletions in *FHIT* than by nucleotide substitutions.

TUMOR SUPPRESSION AND CELL DEATH ON REEXPRESSION OF FHIT PROTEIN

Lung (4), stomach, kidney, and gastric (5) cell lines containing *FHIT* deletions, stably transfected with retroviral *FHIT* constructs or infected with adenoviral (6) *FHIT* constructs show suppression of tumorigenesis concomitant with induction of apoptosis. Because lung epithelia are exposed to carcinogens and the *FHIT* locus is fragile, inactivation of *FHIT* in preneoplastic lesions may allow survival of damaged cells and allow cancer progression in response to further genetic changes. Thus, strategies that kill *FHIT* cells before they become malignant might greatly reduce cancer incidence.

Fhit KNOCKOUT MICE

The murine *Fhit* gene, encoded at 14A2, a location syntenic with human 3p14.2, also spans a fragile site (7,8). An embryonic stem cell was subjected to targeted disruption of *Fhit* and animals heterozygous for germline *Fhit* inactivation were obtained. When subjected to intragastric doses of nitrosomethylbenzamine, heterozygous animals developed stomach and sebaceous tumors that resembled human Muir-Torre syndrome (9), a familial cancer syndrome thought to result from deficiencies in mismatch repair. These observations suggest that maintenance of the *Fhit* gene or Fhit protein expression may depend on an intact mismatch repair system.

Fhit ENZYMOLOGY AND PROTEIN STRUCTURE AND RELATIONSHIP TO CELLULAR FUNCTION

Fhit protein is a dimer with two identical binding sites for Ap_nA (10), a family of low-abundance nucleotides that accumulate in response to interferon and contact inhibition of growth. Fhit protein binds ApppA and AppppA with nearly equal affinity (11). The His96Asn allele of Fhit, which binds Ap_nA well but is greatly defective in hydrolysis, is functional in tumor suppression (5,12). Thus, the Fhit-Ap_nA complex is hypothesized to be the active signaling form. Nucleotide hydrolysis and product release may terminate the ability of Fhit to form a complex with a proapoptotic effector. The crystal structure of Fhit bound to nonhydrolyzable ApppA, solved at 2.6 Å resolution, showed that binding of ApnA substrates converts a concave, positively charged surface in the Fhit dimer to a convex surface filled with nucleotide phosphates (10).

Fhit INTERACTING PROTEINS

Although the effectors for the proapoptotic activity of Fhit are not known, it has been observed that Fhit homologs in invertebrates are fused to members of the nitrilase superfamily (13). The worm NitFhit crystal structure shows