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Human hyaluronidases map to a candidate tumor suppressor locus

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Abstract. Eukaryotic hyaluronidases are widely distributed, but until recently the only vertebrate enzyme to be cloned was the sperm-specific enzyme, PH20. We have now purified the hyaluronidase of human plasma. The identical enzyme, as well as a second proteolytically processed form, is present in urine. Amino acid sequence of the purified hyaluronidase matched a cDNA in the human Expressed Sequence Tag database which, in combination with 5'-RACE-PCR, was used to clone the gene, termed HYAL1, coding for a protein of 435 amino acids. HYAL1 is identical to an uncharacterized gene positionally cloned by others at chromosome 3p21.3 that is homozygously deleted in several small cell lung carcinoma cell lines. We have also identified two additional paralogous hyaluronidase-like genes flanking HYAL1 on chromosome 3p21.3 termed HYAL2 and HYAL3. We are evaluating the candidacy of these two genes as potential tumor suppressors. The mouse hyaluronidase gene, by convention termed Hyal1, was also cloned and expressed and found to be 73% identical to the human enzyme. In mouse, serum hyaluronidase polymorphism has previously been mapped to 60 cM from the centromere of chromosome 9, which corresponds to a cytogenetic location of 9F1-F2, a region syntenic to the human 3p21.3. Mice with alleles of Hyal1 producing higher levels of plasma hyaluronidase have greater resistance to transplanted tumor growth. Products of these candidate tumor suppressor genes are being evaluated as potential anticancer agents.

Keywords. Hyaluronidase; chromosome 3p21.3; tumor suppressors.

1. Introduction

This laboratory has been investigating the biology of hyaluronic acid (HA, hyaluronan), and most recently, the catabolism of this glycosaminoglycan (GAG) by enzymes termed hyaluronidases. Despite their relative importance, this is a poorly understood family of enzymes, that until now have been relatively neglected[1,2]. We undertook purification of some of these activities, in order to characterize the enzymes involved in HA catabolism[3]. When the plasma and urine enzymes were cloned and sequenced[4,5], they were found to be products of a single gene that mapped to a region previously recognized as a candidate tumor suppressor site, 3p21.3[6]. The mouse enzyme mapped to the orthologous chromosomal region, 9F1-F2[6], and had also been previously identified, by classical genetic means, as a site that correlates with tumor suppressor activity[7]. HA promotes tumorigenicity. Loss of some hyaluronidase activities, therefore, may promote growth and

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spread of certain human malignancies. Furthermore, administration of testicular hyaluronidase decreases tumor incidence and tumor size in mice treated with a chemical carcinogen, DMBA [8]. All of these observations support the suggestion that hyaluronidase may be a tumor suppressor gene product.

Previous cytogenetic investigations have independently documented frequent deletions and structural rearrangements of 3p in a number of human malignancies. Allelic loss of 3p21.3 is among the most frequent of such occurrences in malignant disease, particularly in lung tumors [9–12]. Here, we review the evidence that plasma hyaluronidase may be the product of one of the tumor suppressor genes at that locus.

2. The HA substrate

The predominant substrate for the hyaluronidases, HA, is a straight chain glycosaminoglycan (GAG) polymer, containing repeating disaccharide units of [D-glucuronic acid (1-β-3) N-acetyl-D-glucosamine (1-β-4)]$_n$. It is the simplest of the GAG's, the only one which is not covalently linked to a core protein, and the only naturally occurring GAG that is not sulfated. Several HA synthase genes have recently been identified [13]. Synthesis of HA occurs on the inner side of the plasma membrane of cells [14], and is then extruded by an unknown process onto the cell surface. HA is ubiquitous in the extracellular matrix (ECM) of animal cells, but is particularly prominent in the ECM of cells undergoing rapid turnover, as in embryogenesis, tissue repair, wound healing, and carcinogenesis. It also surrounds undifferentiated pluripotent stem cells [15]. HA promotes cell motility, adhesion, and proliferation [15–17]. All of these processes require cell movement and tissue organization. The tight regulation required of HA expression under such conditions is modulated in part by HA receptors such as CD44 [18–20] and RHAMM [21,22].

HA can occur in a number of physiological states, circulating in a relatively free form in lymphatics and in the circulation and also fixed to cells and tissues. Most of this "fixed" HA is in equilibrium with the circulating HA and is easily dissociated. Both circulating and "fixed" HA can be bound to proteins termed hyaladherins [23,24]. The HA can also be very tightly associated with proteoglycans such as aggrecan and link proteins in the ECM of cartilage [25]. The Km of such associations are of such magnitude that HA is not easily dissociated and is not in equilibrium with the HA of the surrounding loose connective tissues. HA also occurs covalently bound to proteins such as inter-alpha trypsin inhibitor [26].

3. HA promotes tumor growth

These associations of HA in the ECM and in the pericellular matrix occur normally, but levels are markedly elevated during embryogenesis, in wound healing, whenever rapid tissue turnover and remodeling are occurring and in the process of carcinogenesis. HA takes on a large volume of water of hydration, expanding tissue spaces, creating an environment that facilitates cell movement, and provides easy access for invasive cells. During wound healing and early on in the inflammatory process, the high levels of HA facilitate entry of polymorphonuclear leukocytes. The HA creates a hydrated environment, accounting for the initial edematous swelling, pushing compacted resident cells apart, enabling inflammatory cells to remove debris and to mount a first line of defense against microorganisms. The HA-rich environment accounts for the motility of

cells during early embryogenesis when cell movement and cell proliferation are particularly prominent[15]. The process of tumor cell invasion and metastatic spread recapitulates these early HA-rich stages of both wound healing and embryonic growth. The HA, when in excess, facilitates invasion by swelling and disrupting tissue integrity so that cell–cell interactions are prevented, and cell receptors and their ligands no longer have access to each other, and creating spaces for cell movement. Thus, tumor-associated HA provides an extracellular matrix that facilitates invasion[27], and in many cases, HA in the environment of tumor cells correlates with metastatic behavior[28].

4. The acid-active hyaluronidase in human plasma

When whole serum is acidified, HA is rapidly degraded, demonstrating that serum contains a circulating lysozomal-like hyaluronidase activity[29,30]. This does not occur at neutral *p*H. Initially, isolation of hyaluronidase was attempted in this laboratory, with a 5,000-fold increase in specific activity[3]. Precise purification could not be calculated because of the simultaneous elimination of several apparent inhibitors of acid-hyaluronidase activity. A single protein band was obtained, that upon sequence analysis was identical to hemopexin[31,32]. We are not able to conclude with confidence that certain isoforms of hemopexin do indeed contain hyaluronidase-like activity. However, it became apparent that there was contained, within that single band, minute amounts of a hyaluronidase enzyme. This is a protein present in minuscule amounts, with very high specific activity, which is unstable in the absence of detergents. This protein was successfully purified and cloned and found to be 40% identical in amino acid sequence to PH-20[4]. Monoclonal antibodies were prepared specific for the plasma enzyme. Immunoprecipitation removed all acid-active hyaluronidase activity, suggesting that there is only one such enzyme in human plasma.

5. Mouse plasma hyaluronidase

We have now, in addition, cloned the mouse ortholog of human plasma hyaluronidase and determined the chromosomal location of the gene in human and mouse. The gene maps to chromosome 3p21 in human and a syntenic region[33,34] on chromosome 9F1-F2 in mouse[6].

By convention, the plasma enzyme in the human is termed HYAL1, the plasma enzyme in the mouse, Hyal1. A candidate tumor suppressor gene at 3p21.3 has been designated LuCa-1, an acronym for Lung Cancer-1, since it has been implicated in the etiology of many lung cancers. The sequences coding for HYAL1 and LuCa-1 are identical. The two sites adjacent to HYAL1 containing hyaluronidase-like sequences, are referred to as HYAL2[35] and HYAL3, as well as LuCa-2 and LuCa-3, respectively.

6. Hyaluronidase in human urine

An acid-active hyaluronidase in human urine has been identified for many years[36–38]. Isolation of this activity revealed that it was identical to the plasma enzyme[5]. However, a second band was observed that was not present in plasma. This second band of 45 kDa was an enzymatically processed form of the 59 kDa protein. This was not an example of zymogen processing of an enzyme to an active form, as is found with many proteases, since both forms of the enzyme had activity. The specific activity of the two isoforms are

comparable. Rat liver lysozomes also contain two forms of hyaluronidase[39]. The lower molecular weight form of urinary hyaluronidase may also be of lysozomal origin. This second processed form may be analogous to the two forms of PH20 that have been identified[40,41]. The higher molecular weight PH20 is membrane-bound, and the processed second activity is a soluble form of that enzyme. It has not yet been established whether the two forms of the urine enzyme parallel the two forms of the sperm-associated enzyme. However, it is of intrinsic interest to note that an enzyme that degrades chitin, chitotriosidase, found in monocytes and macrophages, is processed in an analogous manner[42]. Chitin, a β-linked polymer of N-acetylglucosamine, has structural similarity to HA.

7. Polymorphism of mouse plasma hyaluronidase – Levels correlate with tumor resistance

In mouse, segregation analysis by an interspecific backcross of BALB/c × C57BL/6 strains has shown that serum hyaluronidase level polymorphism is linked to a single locus on chromosome 9, which was designated Hyal1[43]. This locus was subsequently refined to 60 cM from the centromere of chromosome 9[44], which corresponds to a cytogenetic location of 9F1-F2[34]. Therefore the mouse Hyal1 gene we have cloned is very likely to be the gene responsible for the hyaluronidase polymorphism mapped to this locus.

Congenic mouse strains differing only at the Hyal1 locus have been used to investigate the effect of serum hyaluronidase levels on transplanted tumor growth[7]. Mice with the allele producing higher levels of circulating hyaluronidase have greater resistance to tumor growth than mice possessing alleles that produce lower levels of hyaluronidase[7].

8. Hyaluronan around tumor cells correlates with tumor aggressiveness

The proposed mechanism of the suppression of tumor growth in mice is that removal of the pericellular coats of HA that surround tumor cells by hyaluronidase renders the tumor more accessible to cytolytic cells[45,46]. HA is frequently present in elevated amounts around tumor cells, and it is presumed to facilitate tumor-cell migration and proliferation[27,28,47]. There is evidence for a direct relationship between levels of HA and malignancy and invasiveness. This was first noted by Ozzello *et al* who observed the growth-promoting effect of glycosaminoglycans on mammary carcinomas[48]. Subsequently Toole and coworkers observed that the HA content of invasive V2 rabbit carcinoma was 3 to 4 times greater than that of the same tumor when grown under non-invasive conditions[49].

Significant aberrations in the localization of hyaluronidase have been reported in various carcinomas[50,51], and human serum hyaluronidase levels drop markedly in neoplasia[52]. However, since mutations or other aberrations in HYAL1$^{(LuCa\text{-}1)}$ have not yet been reported in small cell or other carcinomas, the evidence that hyaluronidase may be involved in suppression of tumor growth is indirect. Furthermore, the experiments of Fiszer-Szafarz and others may only indicate linkage disequilibrium between an allele producing elevated levels of hyaluronidase, and another closely-linked gene that has tumor suppressor function, since there are several other candidate genes at this locus[53].

9. The 3p21.3 locus in human cancer

Genetic alterations on the short arm of chromosome 3 are among the most frequent in human malignancies. They occur with particular frequency in lung tumors and other

tobacco-related malignancies[9–12]. Three distinct regions on 3p have been identified containing alterations[54], but at 3p21.3, homozygous deletions have been observed with the greatest frequencies, indicating the presence of one or more tumor suppressors. Most cancers of the oral cavity, head and neck, and of the lung are associated with tobacco use. Lung cancers are predominantly bronchogenic carcinomas or small cell carcinomas, the latter arising from neuroendocrine cells. They are rapidly invasive and have a poorer prognosis. Deletions of 3p21.3 are found in many lung tumors, but occur most frequently in small cell carcinomas and the cell lines derived from such tumors[54–59].

Cytogenetic investigations have revealed frequent deletions of 3p in malignant mesothelioma, a rare mesodermally derived tumor[60]. Exposure to asbestos has been implicated as a major factor in the etiology of this tumor[61]. Many of these tumors appear to arise from toxic exposure, and an aberration at the 3p21.3 locus would provide an attractive parallel with the tobacco-induced malignancies. A number of cell lines have been examined, and a lesion in 3p has been documented. Some tumor lines have aberrations at several loci, which is most consistent with a large chromosomal region being deleted. However, specific 3p21.3 deletions are also observed.

Nasopharyngeal carcinoma (NPC) is particularly prevalent among Chinese of Cantonese origin. Several factors have been implicated in the etiology of NPC, including Epstein-Barr virus, genetic factors that contribute to a predisposition, as well as environmental exposure. Cheng *et al*[62] used a monochromosome transfer method followed by detailed cytogenetic analysis. This is a technique of adding chromosome fragments to tumor cells, which then suppresses tumorigenicity. At least three loci of tumor suppressor activity are identified on chromosome 3p, but the analysis of Cheng et al. by monochromosome transfer demonstrates that functional activity is localized to 3p21.3. This confirms previous LOH studies in NPC[63].

10. Conclusion

Despite an intense search to identify the putative tumor suppressor that resides at the 3p21.3 locus, no firm candidate has yet been identified. This is because until recently the candidate region was only narrowed to a very "gene rich" 300 kb contig[64]. This necessitates an intense and enormous search to identify mutations in candidate genes. However, more recently, a 30 kb homozygous deletion has been identified in a small cell lung carcinoma line[65], and an overlapping 220 kb homozygous deletion has also been identified in a breast cancer cell line[66]. This greatly narrows the area of search for mutated genes. HYAL1 and HYAL2 fall within these two recently identified homozygous deletions, and therefore are strong candidates. But no mutations have yet been identified in these genes. However, one cannot rule out the possibility that non-mutational inactivation of one or both of these genes is occurring. This may occur by an epigenetic effect, such as methylation of CpG islands, or down-regulation of translation. We are currently investigating these possibilities.

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