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Short Communication

Alpha-Fetoprotein Uptake and Cytoplasmic Trafficking in Cancer and Immune-Associated Cells: Relevance to Adaptive Immunity

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Abstract

The cell uptake and trans-passage of alpha-fetoprotein (AFP) through the cytoplasm has long been reported in the literature in a progressive albeit piecemeal fashion. However, recent novel immunohistochemical studies have provided valuable new insights into the intra-cytoplasmic fate of endocytosed AFP in various cells including cancer and immune-responsive cells. Employing biochemical inhibitors and antibodies to various endosomal and cytoskeletal-related proteins and enzymes, investigators have tracked in detail the organelle pathway from AFP cell uptake to its final intracellular compartmental destination. These added contributions highlight and contrast two different cytoplasmic trans-passage routes for AFP. One pathway involves endosomal vesicular transport of AFP free of ligands, while the other route consists of ligand-bound (fatty acids/steroids) AFP as a component of an in-and-out cell shuttle system. Finally, the present commentary distinguishes between antigen-presenting dendritic cells employing the scavenger mannose receptor (CD206), and hepatoma (HEPG2) cells utilizing the CD36, LOX-1, and SRB1 scavenger receptors.

Keywords: *Alpha-Fetoprotein; Dendritic Cells; Immunity; Antigens; Presentation; Cells; Receptors; HEPG2*

Introduction

The uptake of human Alpha-fetoprotein (HAFP) has been under investigation for many years revealing the presence of multiple cell surface receptors which have been identified, purified and characterized. Early uptake studies on the receptor-mediated endocytosis of AFP within coated pits of the plasma membrane have been reported in which AFP was generally tracked to vesicles, multi-vesicular bodies, and the trans-Golgi network adjacent to the nucleus [1,2]. Subsequent studies have shown that the uptake property of AFP was present in both non-neoplastic cells and malignant cells including breast carcinomas, hepatomas, neuroblastomas, Lymphomas, T-and ß-cell malignancies; the non-malignant cells include, lymphoreticular cells, fibroblasts, dendritic cells, and activated T-lymphocytes [3-6]. These studies strongly support the presence of multiple and diverse AFP cell- surface receptors present as integral cell membrane proteins. In overview, the cellular uptake of AFP has been well-established as a receptor-mediated endocytotic process which has been demonstrated and identified by means of physiochemical properties such as binding association constants, number of binding sites, molecular structure and weight, and antibody production. The object of the present commentary is to compare and contrast the mechanism of AFP uptake in immune-associated cells versus hepatoma cells via protein uptake by various scavenger receptors (SRs).

AFP and the Mannose Receptor

A significant finding in recent literature concerns the identification and characterization of the mannose receptor (MR; CD206) of dendritic cells as the primary uptake and endocytotic agent for both umbilical cord and tumour-derived HAFP [7]. This observation constituted yet another confirmatory report in the biomedical literature that AFP binds and complexes with a specific scavenger receptor (CD206) wherein the complex fuses with an endosomal vesicle, and migrates through the cytoplasm to ultimately reside in the intracytoplasmic perinuclear compartment. The mannose receptor (MR) is a C-type lectin, type-I transmembrane protein present largely on macrophages and dendritic cells, but lacking on monocytes [8-10]. MR has also been localized on the surface of fibroblasts, keratinocytes, and endothelial, brain, and placental cells. This scavenger-type MR is not to be confused with the transmembrane mannose-6-phosphate receptor, which largely targets enzymes to lysosomal compartments of the cytoplasm. The MR itself plays a role in both innate and adaptive immune responses in vertebrates and in glycan-containing microorganisms [11,12]. The MR functions in the clearance of sulfated glycoproteins especially those bearing a terminal mannose, N-acetylglucosamine (N-AGA), galactose, and fucose residues [13-15]. In comparison, the mature circulating AFP molecule itself is known to contain approximately 4% total carbohydrate moieties which includes N-AGA (1.2%), mannose sugars (2.2%), sialic acid (0.9%), small amounts of glucose [14]. The N-terminal extracellular domain of the MR is composed of a cysteine-rich domain, a fibronectin type-II domain, and 8 consecutive C-type carbohydrate recognition domains (CRD) juxtaposed to the transmembrane region [13,15]. The N-terminal cysteine-rich domain resembles the Ricin-B chain and binds sulfated sugar moieties, N-AGA, and galactose residues. The fibronectin-II domain normally binds and internalizes collagen types I, II, and III into macrophages and liver sinusoidal cells [16]. The 8-repeat CRD domain binds calcium and sugars such as terminal mannose residues. Proteolytic cleaving by metalloproteases of the MR near the transmembrane region and subsequent cell surface shedding produces a soluble form of the MR extracellular domain molecule which then circulates. The cytoplasmic tail domain of the entire intact MR mediates endosomal sorting through a di-aromatic amino acid (Tyr-Phe) motif, essential for rapid cell internalization.

The cell surface MRs reside within clathrin-coated pits juxtaposed to lipid rafts which constitute cholesterol and glycosphingolipid-enriched microdomains of the bilayer cell membrane. The MRs reportedly recycle continuously between the external surface of the plasma membrane and the clathrin-coated endosomal compartments in the cytoplasm. When either cord-obtained (cAFP) or tumor-derived (tAFP) bind to the cell surface MR, subsequent cell uptake occurs by receptor-mediated endocytosis. Utilizing pre-treatment of DCs with binding inhibitors and blocking agents (mannon, polyinosinic acid, anti-MR antibodies), a previous study determined that AFP uptake in DCs occurs largely by MR receptors and less by pinocytotic processes and other scavenger receptors [7]. In contrast, cAFP and tAFP uptake by cultured liver cancer cells (HepG2) do not utilize the MR, but rather use the scavenger receptor CD36, and to a lesser degree the LOX-1 and SRB1 scavenger receptors. These scavenger receptors have been previously identified and confirmed as AFP receptors present on macrophages, lymphoreticular, and tumor cells [17-19].

Cell Uptake Kinetics

Within 1 to 3 minutes after AFP-to-MR binding to the invaginated plasma membrane vesicles, endocytotic passage in the cytoplasm takes place within clathrin-lined vesicles referred to as receptosomes [20,21]. These vesicles containing AFP-MR bound complexes are directed toward and fuse with early stage endosomes derived from the endoplasmic reticulum (ER) membrane. The subsequent endocytotic transpassage is known to involve tyrosine kinase activation, actin filament assembly/disassembly, adaptin, GTPase dynamin-scission of vesicles, and dynamin recruitment [22]. By means of organelle-specific immunohistochemistry, it was demonstrated that AFP/MR containing vesicles do indeed fuse with early endosomes, but do not with late endosomes, lysosomes, KDEL peptides, or the transreticular golgi constituents of the DC cytoplasm [7]. Within 30 minutes after endocytosis, the early endosomal vesicles (AFP:MR) are shuttled via kinesin and the dynein motor microfilaments of the cytoskeletal system to the perinuclear region; this compartment is known to be continuous with the channels of the ER in the cell cytoplasm [23]. Within the perinuclear compartment, the AFP/MR endosomal vesicle complex (now transformed into late endosomes), undergoes a process which dissolves the endosomal clathrin coating/lining by means

of the clathrin adaptor protein-2 (AP-2) and a tyrosine protein phosphatase termed Auxillin [24]. Once released from the late endosomal vesicle, the MR separates from its AFP-cargo, and the MR is recycled back to the cell membrane for re-insertion into the clathrin-coated pits/lipid raft region of the plasma membrane bi-layer. AFP is retained in the perinuclear compartment either as a stored entity or destined for other activities (see MHC below). For example, it has been proposed that AFP might serve as a "gate" regulator for the entrance of karyophilic transcription factors and/or their inhibitors into the nucleus, thus preventing their import into the nucleoplasm through nuclear membrane pores [25]. To this end, it has been demonstrated that cytoplasmic AFP is capable of blocking retinoic acid-retinoic acid receptor (RAR) nuclear signaling by binding to the retinoic acid receptor in the cytoplasm and preventing RAR trans-nuclear passage [26-28]. If such an event were to occur, it is conceivable that cytoplasmic AFP could block nuclear trans-passage of growth factors from its perinuclear storage location thus indirectly influencing growth regulation in tumor cells.

Antigen Presentation

Concerning antigen presentation, reports have indicated that the MR binds and internalizes both cord-and tumor-derived AFP. This cell uptake event exemplifies the role that MRs plays in immature dendritic cells for activating the adaptive branch of the immune response [7]. After binding to the MR and passage to the perinuclear compartment, certain mannosylated protein antigens (such as AFP) are transformed into late endosome constituents within the cell for trafficking to the Major Histocompatibility Complex (MHC) processing system intended for proteins and other related antigen presentation molecules [29]. In the Butterfield., *et al.* study [7], it was demonstrated that unbound AFP residing in the perinuclear compartment colocalized with late endosomes, a process known to be consistent with a classical MHC Class-II (not Class-I) processing/presentation of antigens intended for passage to T-lymphocytes.

In the latter report above, it was shown that AFP proteins internalized in DC cells as an AFP/MR vesicular complex were transported and transformed into late endosomal vesicles for processing and loading onto antigen-presenting molecules (i.e. CD1b) [7]. However, mature DCs and macrophages can use the MR pathway for antigen presentation in an alternate manner. A membrane cleaved soluble version of the extracellular domain of MR can bind to antigens and direct them to effector cells in lymphoid organs via its cysteine-rich domain, thus activating the adaptive immune system.

The intracellular tail domain of the MR does not contain a signaling motif, but nonetheless has a passive role for production and secretion of pro-and anti-inflammatory cytokines in the phagocytosis of antigens and pathogens [8,9]. The MR also appears to assist other cell surface receptors (Toll Receptors, NK Receptors) into triggering signal cascades which cause secretion of cytokines such as Interleukins (IL-8 and others). It is presumed that the two receptors (i.e. MR and NK) can cluster and complex together to facilitate signal transduction upon pathogenic or antigenic challenge. A comparable example is known to exist in the scavenger family of receptors in which SREC-1 and SREC-3 act in concert to activate signal cascades through extracellular domain binding of these receptors [29].

Experimentally altered AFP cytoplasmic trafficking

In a previous report, an adenovirus vector was utilized to express full length, native secreted HAFP which contained the N-terminal 19-amino acid signal sequence region [7]. A second version of the adenovirus-expressed AFP was also produced which lacked the signal sequence, but incorporated the green fluorescent protein (GFP) into the AFP molecule. The cell uptake pathways of the vector-produced native AFP followed an immediate cytoplasmic entry via a non-endosomal (diffuse-staining) pathway in which AFP ultimately accumulated in the perinuclear compartment. In comparison, the GFP-AFP was also visualized in a diffuse staining pattern throughout the cytoplasm but lacked perinuclear localization. Alternatively, the uptake of circulating AFP bearing fatty acid/steroid cargos into cells involves a well-documented shuttle system for endocytosed AFP which results in re-cyclization and exocytosis of AFP via processing through the trans-reticular region of the Golgi apparatus followed by secretion (exocytosis) to the cell exterior [5,30,31]. However, this system does not apply when DCs are transfected with adenovirus-AFP and GFP-AFP. In comparison, the non-signal sequence- containing GFP-AFP can be viewed throughout the cytoplasm producing a diffuse cytoplasmic staining pattern in addition to localization in the Golgi apparatus and in trans-reticular Golgi regions [7]. Thereby, the lack of cell secretion of the GFP-AFP may be explained by obstruction and/or lack of a signal sequence on the N-terminal region of the AFP molecule.

The AFP vaccination potential

Previous studies by Butterfield., *et al.* reported the amino acid sequence of multiple immunodominant epitopes derived from full-length native AFP which could potentially serve in vaccine development; such sequences included over-lapping sequences $AFP_{137-145}$ and $AFP_{158-166}$ and a latter sequence $AFP_{325-334}$ [32,33]. The immune cytokine (IL-2, INF-α, TNF) production response was stimulated by all 3 antigenic epitope sites with $AFP_{137-145}$ showing the highest response frequency. However, the highest responses for $AFP_{158-166}$ and $AFP_{325-334}$ were only obtained after stimulation and production by adenovirus- and GFP-AFP exposure. IL-2 secreting $CD8^+$ T-cells and $AFP_{137-145}$ and $AFP_{158-166}$ - cytokine producing cells were increased when MR-mediated AFP uptake was inhibited by anti-MR antibodies. Such activities were found using both cAFP and tAFP preparations, although tAFP had a slightly less effect on CD4 + T-helper cell-producing cytokines than on CD8 T-cells. The helper cell CD4 response showed higher cytokine secretion frequencies when DCs endocytosed full-length AFP than when cytoplasmic expression of adenovirus- and GFP-AFP occurred. This was also true when AFP was secreted after shuttling through the Golgi apparatus. Blockage of MR binding and uptake of AFP was found to reduce the cytokine activation responses indicating that, unlike the MR, other scavenger receptor-mediated endocytosis events do not readily support antigen routing enroute to MHC presentation sites. Thus, MR-mediated routing is more efficient when either intact full length cAFP or tAFP was employed. It can be proposed that MRs and scavenger receptors could endocytose AFP and present AFP-antigens to $CD4^+$ T-cells during fetal development and in the perinatal period. In adult cancer patients with T-cells expressing AFP, it was shown that cAFP and tAFP are both endocytosed by DCs bearing MR receptors and to a lesser extent other SRs; however, some SRs are more highly expressed than MRs on certain tumor cells [7].

The scavenger receptors detected on tumor cells are unlike the DCs in that the SRs are highly involved in the uptake of polyanions from the blood circulation. The polyanionic substances taken up by SRs include modified low and high-density lipoproteins, polynucleotides, polyinosinic acids, lipoic acids, extracellular matrix proteins, and chemically modified/conformationally-altered glycoproteins such as AFP and alpha-albumin [33]. The uptake and internalization of SRs and their bound proteins differ from the antigen presentation role of MRs in that ligand binding to other SRs involves linkage to G-coupled receptor activation that lead to signal transduction, cell-to-cell adhesion, and inner cell membrane focal adhesion complex formation [34,35]. Ligand binding to such SRs can initiate growth responses in macrophages, monocytes, dendritic, and tumor cells. SR binding to their ligands further result in a rapid G-coupled protein response, a rise in intracellular free $Ca^{\#}$ ions, and activation of protein Kinase-C (PKC) together with increased activity of phospholipase-A2 [36]. These latter activation processes induce the internalization of lysophosphatidylcholine from the cell membrane, resulting in apoptosis. Once PKC activation occurs, tyrosine kinase phosphorylation is increased in the initiation of signaling cascades and in protein phosphorylation pathways [37]. Increased phosphorylation of phospholipase - C Gamma-1 and phosphoinositol lead to increased levels of the tyrosine kinase termed “Lyn” involved in cell cycle, DNA synthesis, and anti-apoptotic events [38]. Some SRs, such as CD36, also activate NFkB and IKK kinases and the secretion of TNF-alpha, and IL-6 cytokines.

Concluding Statements

It can be deduced from the above discourse that the cell uptake and trafficking of AFP through the cytoplasm can follow either of two diverse and circuitous pathways. Once such pathway involves the endosomal cytoskeletal-transport network; the other is a drop-cargo shuttle system used for circulating carrier protein cell depositions. In the former pathway, AFP is taken up by a receptor-mediated endocytosis procedure after which the AFP: Receptor complex fuses with early endosome vesicles. The fused AFP receptor-containing endosome traverses a cytoskeletal-guided trafficking (transport) network involving a host of enzymes and scaffolding proteins. These entities encompass and include tyrosine kinases, GTPase dynamin, actin filaments, adaptin, kinesin, and dynein motor microfilaments. The final destination of this pathway leads to the cytoplasmic perinuclear compartment wherein AP-2 and Auxillin serve to release the AFP: receptor complex from its enclosed endosome (now referred to as a late endosome). The freed receptor is re-cycled back to the cell membrane and the released AFP is stored within the perinuclear compartment. At this point, AFP is available to be processed within the MHC-Class II system leading to proteolysis and subsequent antigen presentation to T-Lymphocytes at the dendritic cell surface.

An alternate pathway following AFP cell uptake involves a carrier protein-cargo drop-off system mediated through organelle structures such as the trans-reticular Golgi network, lysosomes, and endoplasmic reticulum (KDEL) transport systems. In these cytoplasmic organelle networks, blood carrier proteins (i.e., AFP) can transport and drop off cargos such as fatty acids/steroids into cells for nutrition and growth during development. In summary, the route of AFP delivery into cells can impact either immuno-stimulatory or growth enhancement effects depending on the cell type and ligand-bound status of the carrier fetal protein.

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Conflicts of Interest

The author declares that there are no known conflicts of interest in the preparation of this manuscript.

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