[Frontiers in Bioscience S3, 341-351, January 1, 2011]

Hsp60: molecular anatomy and role in colorectal cancer diagnosis and treatment

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1. ABSTRACT

Quantitative changes in Hsp60 during the development of some tumors suggest that this chaperonin plays a role in carcinogenesis. A description of the specific role(s) of Hsp60 in tumor-cell growth and proliferation is still incomplete, but it is already evident that monitoring its levels and distribution in tissues and fluids has potential for diagnosis and staging, and for assessing prognosis and response to treatment. Although Hsp60 is considered an intramitochondrial protein, it has been demonstrated in the cytosol, cell membrane, vesicles, cell surface, extracellular space, and blood. The knowledge that Hsp60 occurs at all these locations opens new avenues for basic and applied research. It is clear that elucidating the mechanisms by which the chaperonin arrives at these various locations, and characterizing its functions in each of them will provide information useful for understanding carcinogenesis and for developing diagnostic and therapeutic tools for clinical oncology. Some of these issues pertinent to colorectal cancer (CRC) are discussed in this article.

2. INTRODUCTION

The chaperoning system is composed of several groups of molecular chaperones and their cochaperones and cofactors, and other functionally associated molecules (1). This system, essentially involved in maintaining protein homeostasis and in anti-stress mechanisms, is conserved throughout evolution and is present in prokaryotes (bacteria and archaea) and eukaryotes (2).

The chaperoning system can be viewed as the predecessor of the immune system, which is present only in multicelullar eukaryotes. The immune system is also involved in defence mechanisms against foreign invaders and as such it has points of contact with the chaperoning system. In fact, it is now known that the chaperoning and the immune system interact at various levels (1, 3).

Many chaperones but not all are heat-shock proteins (Hsp). Conversely, many but not all Hsps are chaperones. This distinction is generally ignored in the

literature, hence we will use in this article the terms Hsp and chaperones interchangeably.

In the last two decades, new roles distinct from participation in protein folding, refolding, translocation, and degradation, have been ascribed to Hsp-chaperones, for example, in regulation of the innate immune system, gene expression, cell differentiation, DNA replication, and signal transduction, and participation in programmed cell death, cellular senescence, and carcinogenesis (4-7). In addition, also in the last few years it has become evident that defective chaperones can cause diseases, and a new area of medicine and pathology has been defined to encompass the pathologic conditions, the chaperonopathies, in which chaperone malfunction is an etiologic factor (8).

Among chaperonopathies are some types of neoplasms in which chaperones are not defective but are "collaborating" with the tumour rather than with the host. For this reason, these neoplasms have been called "chaperonopathies by collaborationism" or "by mistake" (9). In these tumours, the cellular levels and expression of some Hsp-chaperones are higher than in normal tissues, a fact that may be of some diagnostic and prognostic potential, as well as of value for assessing response to treatment (10, 11).

Last but not least, because of the Hsp-chaperones involvement in carcinogenesis, a modern approach to fight cancer is to develop strategies directed to these molecules, either to inhibit or eliminate them if they are involved in promoting tumor growth, or the reverse, to augment them if they enhance apoptosis (*i.e.*, death) in tumor cells (12, 13). In this regard, anti-chaperone antibodies may be useful tools for identification of tumor cells with chaperones in their surface, and for targeting these tumor cells to deliver to them therapeutic compounds (11).

In this minireview, we focus our attention on the role of Hsp60 in normal cells and during human carcinogenesis, particularly in regard to colorectal cancer (CRC). Many studies performed in various laboratories, among which ours have tested the hypothesis that Hsp60 is a major player in the development of CRC, one of the most frequent malignancies in the Western World (14-20). In this paper, we discuss briefly some recent data while opening a debate on the central role of Hsp60, and other Hsp-chaperones, in carcinogensis and tumor management from diagnosis to treatment, including assessing prognosis and response to medication.

3. HSP60: MOLECULAR ANATOMY AND PHYSIOLOGY

Hsp60, also called chaperonin 60 (Cpn60), is classically considered an intramitochondrial molecule, residing in the matrix in which it works together with its co-chaperonin, Hsp10 (Cpn10) (21, 22). Hsp60 is a 60 kDa protein constituted of three domains: apical, intermediate, and equatorial. Inside mitochondria, it forms a heptamer with the shape of a ring (23, 24). Two rings join together at their equatorial domains and form a barrel with a central cavity inside which the folding of client polypeptides occurs. Hsp10, a 10 kDa molecule, also forms a heptameric ring, which joins the Hsp60 double-ringed barrel, at the apical domain, to occlude the barrel and, thereby, create a closed chamber for polypeptide folding. The binding of the homodecatetrameric barrel with the homoheptameric Hsp10 ring and with seven ATP molecules is crucial for the chaperoning machine to assemble correctly and function, so the end result is the release of mature client proteins, with their correct tridimensional structure, i.e., native, functional conformation (23, 24). In human cells, Hsp60 can also function as a single homoheptameric ring (25-27). Furthermore, the Hs10 ring, the barrel lid, does not seem to be required for the folding of many proteins since their maturation is not affected by inhibition of Hsp10 (28).

In human cells, both the Hsp60 (*HSPD1*) and Hsp10 (*HSPE1*) genes are in chromosome 2, head-to-head, with a common promoter between them (29). The products of the two genes, Hsp60 and Hsp10, are translocated to mitochondria. The Hsp60 amino-acid sequence has a mitochondrial signal sequence that is cleaved when the chaperonin molecules enters the organelle (30). Since both these proteins are highly conserved during evolution (e.g., they are present in all bacteria and some archaea), and since, according to the endosymbiotic theory, mitochondria derive from bacteria, it has been postulated that the fragment of DNA containing the *hsp60* and *hsp10* genes "migrated" from bacterium/mitochondrion to nucleus in an ancestral era (31). Thus, the genes' products developed the capability of reaching the organelle navigating through the crowded cytosol. In this process, evolutionarily very significant, Hsp60 and Hsp10 most likely acquired new functions, for instance, interaction with molecules involved in apoptotic pathways, and with other mechanisms such as those involved in cytoprotection. It is, therefore, not surprising that Hsp60 and Hsp10 are found in extramitochondrial sites, such as cytosol, peroxisomes, other vesicles, cell membrane, etc (22, 32, 33).

The presence of Hsp60 in the cytosol may be due to mitochondrial release, after a pro-apoptotic stress for example, or to cytosolic accumulation due to gene overexpression (34, 35). The two possibilities are not mutually exclusive, and cytosolic Hsp60 molecules released from mitochondria can in principle be distinguished from those that never went into the organelle because the latter have the mitochondrial localization signal whereas the other do not.

The presence of Hsp60 in the cell membrane and in cellular vesicles probably indicates that the chaperonin is on its way to be released into the extracellular space and, from there in some cases it reaches the bloodstream (36-38). Hsp60 may be secreted alone or bound to other molecules, and it may also have an extracellular function (1, 3, 39, 40). It has been postulated that the presence of Hsp60 in extracellular fluids represents an alarm signal for

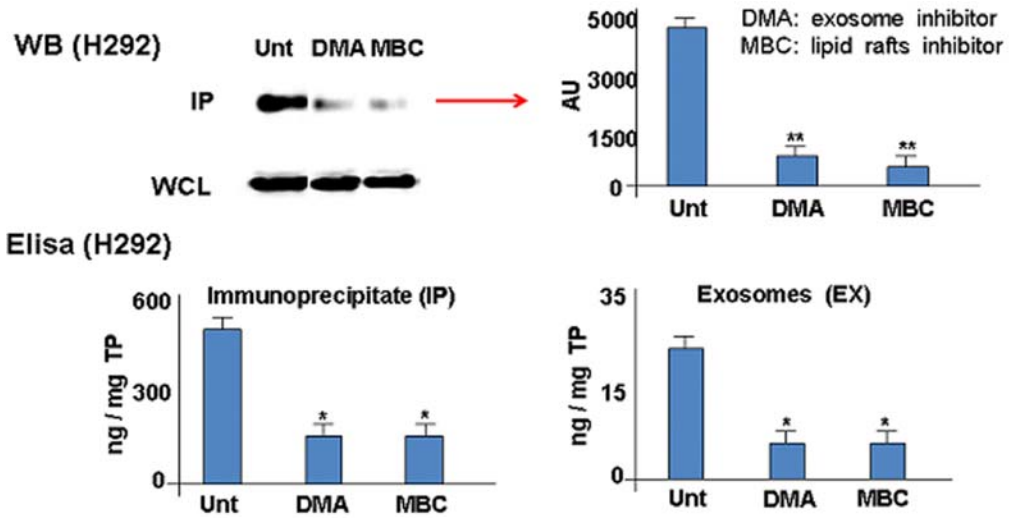

Figure 1. Effect of protein-secretion inhibitors on Hsp60 secretion by tumor cells. A) Hsp60 and Hsp70 detected by Western blotting in: (a) immunoprecipitates from conditioned media from untreated (Unt) and inhibitor-treated H292 tumor cells; and (b) whole-cell lysates from H292 cells. The inhibitors are listed on top of the respective lanes. Histograms to the right represent the levels of the Hsps in immunoprecipitates determined in three separate experiments as mean percentages +/- SD of arbitrary units (AU) obtained with NIH image J 1.40 analysis software. * and **, significantly different from untreated control, $p<0.005$ and $p<0.001$, respectively. The two inhibitors (listed below the bars) significantly decreased secretion of Hsp60 and Hsp70. Also, the data from whole-cell lysates show that the protein-secretion inhibitors had no detectable effect on Hsp levels inside the cells. B) Hsp60 levels secreted by the H292 tumor cells before and after exposure for 1 hour, followed by a 4 hours recovery period, to protein-secretion inhibitors measured by ELISA in: (a) conditioned media; and (b) exosomes. Histograms represent Hsp60 levels expressed as pg of protein normalized for mL normalized for 10^6 cells. Data represent mean +/- SD of three different experiments in duplicate. * Significantly different from untreated control, $p< 0.005$. The results, which are in agreement with those obtained by Western blotting, show that the inhibitors tested significantly reduced secretion of Hsp60 by the H292 tumor cells. (Reproduced with permission from 38).

the immune system, both the innate and the adaptive, which are thus stimulated to mount a proinflammatory response.

4. INVOLVEMENT OF HSP60 IN PATHOGENESIS

Organisms with a defective Hsp60 (e.g., due to an *hsp60* gene mutation), are prone to develop degenerative diseases since quantitative and qualitative mitochondrial protein deficiencies are deleterious and can cause cell death (41-45).

If we recall a well known axiom of cell biology that states that the more numerous are the functions of a molecule, the higher is the risk that its impairment will determine cellular alterations and, thus, the onset of a disease, we fully realize the potential extent of the pathological alterations that a defective Hsp60 will originate. Thus, a prize paid by Hsp60 acquiring a broad range of functions during evolution is that the variety of diseases it may cause if structurally-functionally deficient is extensive.

It has been postulated that cellular stress can cause post-translational modifications in cytosolic Hsp60 (46). These modifications can be responsible of Hsp60 localization in the cell membrane that, in turn, determines its internalization via lipid rafts, accumulation in multivesicular bodies, and release into the extracellular space via the exosomal pathway (38, 46, 47). We have found that Hsp60 is released into the extracellular space by cell lines *in vitro* and this release involves lipid rafts and exosomes, Figure 1 (38). We hypothesize that exosomal Hsp60 is accompanied by other biological active molecules to be destined to other cells. Therefore, Hsp60-containing exosomes may be considered vectors for intercellular communication. This mechanism could have a role in cancer progression, and in the pathogenesis of other conditions such as inflammatory, autoimmune, and degenerative diseases.

It is also pertinent to recall that many microbes, pathogens and non-pathogenes in the human body, produce Hsp60, which can be released and thus can reach the blood and be recognized as foreign antigens by the immune system. The microbial Hsp60 (named GroEL) is structurally very similar to the human ortholog, so antibodies made against the bacterial GroEL almost always crossreact with human Hsp60. This cross-reaction is most likely at the basis of several diseases with autoimmune components, including autoaggression on cells bearing the chaperonin on their plasma membrane (48). The phenomenon of structural, and therefore antigenic, similarity between the prokaryotic and the eukaryotic Hsp60 is a form of what is known as "molecular mimicry," a phenomenon that has been postulated to be involved in the development of some autoimmune diseases (49).

Table 1. Human tumors with increased Hsp60[1]

System	Tumor	Methods[2]	References
Nervous	Astroglyoma	RT-PCR	53
Haematopoietic	Acute myeloid leukaemia	Flow cytometry	54, 55
	Hodgkin's lymphoma	IHC, WB	56
Digestive	Oral liken planus	IHC	57
	Oesophageal squamous carcinoma	IHC	58
	Gastric MALToma	IHC	59, 60
	Large bowel adenocarcinoma	IHC, WB, cDNA microarray, ELISA, proteomics	14, 16, 17, 19, 20
	HCV-hepatocellular carcinoma	2D-gel electrophoresis	61
Male reproductive	Prostate adenocarcinoma	IHC	62-64
Female reproductive	Exocervical carcinoma	IHC, WB	65-67
	Ovarian carcinoma	IHC	68, 69
	Breast ductal invasive carcinoma	2D-gel electrophoresis	70
Endocrine	Adrenal Cushing tumor	IHC	71
Skeletal	Osteosarcoma	IHC, ELISA	72-74

[1]Based on (11), [2]Abbreviations: RT-PCR; real-time PCR; IHC, immunohistochemistry; WB, Western blotting; ELISA, enzyme-linked immunosorbent assay.

Table 2. Human tumors with increased Hsp10[1]

System	Tumor	Methods[2]	References
Haematopoietic	Mantle cell lymphoma	Microarray, IHC, WB	75
Digestive	Large bowel adenocarcinoma	IHC, WB	15, 16
Male reproductive	Prostate	IHC	76
Female reproductive	Ovarian serum cancer	IHC, WB	77, 78
	Exocervical cancer	IHC; WB	15

[1]Based on (11), [2]Abbreviations: see footnote to Table 1.

Table 3. Human tumors with decreased Hsp60 and Hsp10[1]

Hsp	System	Tumor	Methods[2]	References
Hsp60				
	Nervous	Glioblastoma	Proteomics	79
	Respiratory	Bronchial adenocarcinoma	IHC, WB	62, 80
	Digestive	Tongue carcinoma	IHC	81
	Urinary	Bladder transitional cell carcinoma	IHC	82, 83
		Carcinosarcoma	IHC	84
Hsp10				
	Respiratory	Bronchial adenocarcinoma	IHC, WB	80

[1]Based on (11), [2]Abbreviations: see footnote to Table 1.

5. HSP60 IN CANCER DEVELOPMENT

The pattern of Hsp60 levels in cancer cells varies according to the type and stage of the tumor: in some cases there is no discernible change in the tumor cells in comparison with the normal cell counterparts, but in other cases there are clear modifications, which are typical of the cancer cell. For example, Hsp60 levels commonly increase during some types of organ carcinogenesis, (Table 1), and this is often related to a concomitant increase in the Hsp10 levels (Table 2). In contrast, in other tumors, the levels of both Hsp60 and Hsp10 are lower than in normal tissue counterparts (Table 3).

It has not been fully elucidated why and by what mechanism Hsp60 levels increase in some tumors, or decrease in others. We have postulated a positive correlation in lung, tongue, and bladder cancers between exposure to cigarette smoke and reduction of Hsp60 levels and an advanced tumor stage (unpublished).

In what regards the effects of higher Hsp60 levels in dysplastic or neoplastic cells, we suggest that Hsp60 promotes, or at least parallels, tumor mass growth, since Hsp60 in tumor cells is commonly present at higher concentration in cytosol, vesicles, and cell membrane (38, 50). In the cytosol, Hsp60 can bind to pro-caspase 3, blocking its activation after pro-apoptotic stimuli, thus having an antiapoptotic effect, Table 4 (34, 35, 50). Moreover, the chaperonin is secreted from tumor cells via lipid-rafts/exosomal pathways (38), with the potential of playing immunoregulatory roles in the peritumoral environment.

Hsp60 in tumor cells is often localised to the cell membrane, exposed to the outside. This topology makes Hsp60 prone to be recognized by the immune system, e.g., anti-Hsp60 antibodies and/or cytotoxic T cells, which thus have the potential to eliminate tumor cells from the organism (48). Although this antitumor effect can be considered to be an advantageous "natural" antitumoral mechanism, it may also represent one of the ways in which more aggressive clones are selected by escaping antitumor immunity. This could explain why some tumors (in more advanced stages?) are Hsp60 negative

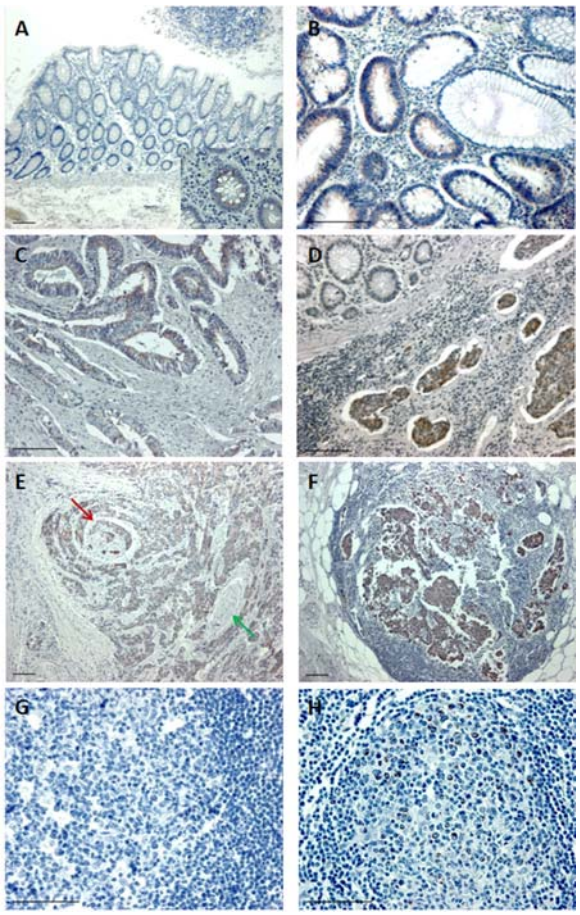

Figure 2. Immunohistochemical assessment of Hsp60 helps to diagnose CRC. A) Hsp60 in normal colon mucosa is under the threshold of detection. Some cells do present spots of positivity inside the cytoplasm suggesting mitochondrial localization of the molecule (inset, original magnification: 100X). B) Dysplastic cells of a low-grade polypoid adenoma, which typically have large nuclei and are tightly packed producing at low magnification the sort of image seen in the left side of the microphotograph, show a diffuse positivity for Hsp60, in contrast to hyperplastic cells (right upper corner), which are usually negative for the chaperonin. C) CRC (colon adenocarcinoma) cells also show a diffuse positivity for Hsp60. The diffuse positivity (here and in dysplastic cells in B) suggests extramitochondrial (cytosolic) localization of the chaperonin. D) Immunohistochemistry for Hsp60 helps to identify islets of tumor cells (right lower corner) underneath a normal mucosa (left upper corner). E) Hsp60 immunostaining show nerves invaded by tumor cells (red arrow) and thus it can distinguish invaded from non invaded (green arrow) nerves. F) Shown is a small colonic lymph node colonized by Hsp60-positive adenocarcinoma cells. G) Image of a hyperplastic follicle in a lymph node exempt from tumor invasion, negative for Hsp60. H) This picture shows a hyperplastic follicle in a lymph node invaded by tumor cells (not shown) that presents a number of lymphoblasts positive for Hsp60. This immunopositivity resembles the punctate pattern observed in normal epithelial cells (A, inset) suggestive of a mitochondrial positivity. Bars: 100 micron.

Some research has been performed to assess the prognostic value of Hsp60 levels in tumor cells, Table 5. The results are encouraging is some cases but are, for the most part inconclusive, due to the limited number of patients studied in depth.

6. HSP60 AND CRC DEVELOPMENT

Hsp60 is a good immunohistochemical marker of CRC (Figure 2). Hsp60 levels, as well as those of its co-chaperonin Hsp10, increase gradually throughout the carcinogenetic steps of CRC, i.e., from normal mucosa through low-grade dysplasia and severe dysplasia to invasive cancer, as shown by immunohistochemistry and Western blotting (14-16). Our results have been confirmed by others (17, 18). *HSPD1* overexpression in colon cancers was also recently shown and bioinformatics data indicated that this gene is one of the best markers for diagnosing these tumors (19). These findings support the potential utility of measuring Hsp60 at both, protein and gene-expression levels, for diagnostic purposes and for assessing prognosis of pre-neoplastic and neoplastic lesions in routine surgical pathology (20).

The histological immunolocalization of Hsp60 and Hsp10 help to discriminate CRC staging, since it provides a means to assess the levels of tumor invasion and infiltration of vessels, nerves, and regional and distant lymph nodes, thus evidencing metastases. We found that higher levels of Hsp60 but not Hsp10 are correlated with higher tumor grade and, thereby with higher tumor aggressiveness. Lymph node metastases, even very small, are also positive to Hsp60, which suggests that testing for Hsp60 will help to detect micrometastasis. Normal parenchyma (especially cortical follicles) of infiltrated lymph nodes presents higher immunoreactivity for Hsp60 than the normal parenchyma of hyperplastic lymph nodes without metastases (16).

An intriguing observation was that CRC tumor-cell lysates from fresh tissues showed in Western blotting two Hsp60 bands, one of them was slightly heavier than the canonical 60 kDa molecule (16). This could be interpreted as that the heavier band corresponds to the Hsp60 with its organelle-localizing sequence still in place, while the smaller molecule corresponds to the same molecule but without the signal sequence. These two types of Hsp60 molecule in the cytosol could be targets of various distinct post-translational modifications, i.e., a mechanism for generating diverse Hsp60 molecules each with a particular function in the cell and/or outside the cell. One of these special, non-canonical Hsp60 locales is, in colon cancer, the cell membrane (51).

Along the same lines, we recently observed Hsp60 in colon mucosa of patients with inflammatory bowel diseases (IBD), i.e., Crohn's disease and ulcerative colitis (52). Both conditions are considered high risk for developing CRC. We found by immunohistochemistry that Hsp60 is increased in both diseases in comparison with normal controls in epithelium and *lamina propria*. It is likely that Hsp60 participates in the inflammatory

Table 4. Effect of Hsp60 on apoptosis in tumor cells[1]

Cells studied	Hsp60 localisation	Interacting molecule	Stimulus	Effect	References
Jurkat	Mitochondria	Pro-caspase 3	Staurosporine	Pro-	85
Jurkat	Free / soluble	Pro-caspase 3	Caspase-6	Pro-	86
H292	Undetermined	Pro-caspase 3	H_2O_2	Anti-	50
PC3, LNCaP, MDAMB231, HCT116	Cytosol (with mitochondrial release)	Pro-caspase 3	BMD-188	Pro-	34
PC3, LNCaP, MDAMB231, HCT116	Cytosol (without mitochondrial release)	Unknown	BMD-188	Anti-	34
MCF-7	Mitochondria	Survivin	Hsp60 knockdown	Anti-	35
MDAMB231, HCT116	Mitochondria, cytosol	p53	Hsp60 knockdown	Anti-	35

[1]Based on (11)

Table 5. Human Hsp60 levels and cancer prognosis[1]

Tumor	Hsp60	Prognosis	References
Acute myeloid leukaemia	Increased	Bad	55
Oesophageal squamous carcinoma	Increased	Better five-year survival	57
Ovarian carcinoma	Increased	Worse in patients treated with cisplatin-containing chemotherapy	68
	Increased	Better	69
Vesical transitional cell carcinoma	Decreased	Bad outcome of local treatments	82
	Decreased	Poor response to neoadjuvant chemoradiotherapy	87

[1]Based on (11)

processes that determine mucosal remodelling and, as a consequence, the chaperonin takes part also in the process of carcinogenesis that often occurs in IBD mucosa, a possibility that is currently under investigation.

7. CONCLUSIONS

The field of chaperonology is now expanding since it is known that Hsp-chaperones are involved in the pathogenesis of a number of diseases, the chaperonopathies, among which some types of cancer. Chaperonotherapy is defined as the utilization of Hsp-chaperones for treating chaperonopathies and other diseases (13). The possibility to restore a normal set of molecular chaperones inside neoplastic cells would be a way to limit the tumor growth if the chaperones have effects, like a pro-apoptotic effect, in the tumor cell that do not favour its growth.

Another aspect of chaperonotherapy is to use Hsp-chaperones as targets to direct anti-tumor reagents to tumor cells. Since Hsp-chaperones are in general strong immunogens, anti-chaperone antibodies are potentially useful reagents to kill tumor cells with one or more Hsp-chaperones on its surface.

As far as Hsp60 is concerned, it has been shown that its levels increase during the carcinogenetic steps in several types of neoplasm. Hsp60 levels determined by immunohistochemistry in tumor biopsies have been found in some cases to positively correlate with better prognosis as compared with the same type of tumors in which Hsp60 levels did not increase. However, more extensive studies are necessary with many patients and matched controls to determine with precision the real diagnostic and prognostic value of Hsp60 levels in tumor cells. In addition, histological determinations should be accompanied by measurements of Hsp60 in the sera of patients, so as to obtain a more complete picture of the participation of this chaperonin in the clinico-pathological spectrum of signs and symptoms that characterize patients with cancer.

In some of those cases in which Hsp60 levels are augmented in tumor cells it is likely that the chaperonin has a pro-tumor effect. Therefore, chaperonotherapy should in this situation aim at eliminating or inhibiting Hsp60. The chaperonin would be the target for anti-chaperone agents.

Hsp60 accumulates in the cytosol of tumor cells and is secreted with involvement of lipid-rafts and exosomes. The latter exosomes represent a way to exchange "content" and thus "information" between cells. Hence, Hsp60 is probably involved in communication between tumor cells. It is not yet clear whether Hsp60-loaded exosomes have pro- or anti-tumor effects; do they stimulate pro- or anti-inflammatory mechanisms in the peritumoral area and thus influence tumor growth?

Hsp60 has been found localised to the cell membrane of some tumors *in vitro* but this observation has not yet been confirmed *in vivo*. Moreover, we do not have enough information about the Hsp60 amino acids that are exposed on the membrane surface; this information would be key to generate anti-Hsp60 antibodies with a potential antitumor effect. We know that some normal cells, such as endothelial cells, under stress (e.g., hypertension) do expose Hsp60 on their membrane and are, therefore, prone to be recognized by anti-Hsp60 antibodies.

In regard to the above, it is important to recall that human microbiota includes many organisms that release Hsp60 in various tissues, particularly during bacterial infections, and the circulating microbial chaperonin can induce an immune response with cells and antibodies that crossreact with the human ortholog. This mechanism of crossreactivity due to molecular mimicry has been implicated in the pathogenesis of a number of diseases with autoimmune components, such as arthritis, diabetes, thyroiditis, and others. The same type of phenomenon may cause also the "natural killing" of tumor (or pre-tumor) cells expressing Hsp60 on their membrane. However, molecular mimicry and crossreactive antibodies could also lead to selection of tumor cell clones that have no Hsp60 on the surface, which would thus escape the action of the immune system.

In summary, we have gathered evidence in the laboratory and with human samples, and from the literature,

which strongly suggests that Hsp60 is involved in tumor development in humans. Hsp60 occurs in the mitochondria of tumor cells, as expected, but it is also present in the cytosol and on the surface of these cells. It is thus obvious that this chaperonin must occupy the central stage in future studies aimed at understanding the mechanisms of tumor cell growth, and in efforts dedicated to the development of antitumor agents.

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Abbreviations: Hsp: Heat shock protein; CRC: colorectal cancer; CPN: chaperonin; IBD: inflammatory bowel diseases

Key Words: Chaperoning system, Chaperonology, Chaperonopathies, Chaperonotherapy, Hsp60, Clinical oncology, Colorectal cancer, Review

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