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Review

Circulating nucleic acids in plasma and serum (CNAPS) and its relation to stem cells and cancer metastasis: state of the issue

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Summary. The presence of circulating cell-free nucleic acids has been demonstrated both in disease and health. In the last decade, a burst of papers about Circulating Nucleic Acids in Plasma and Serum (CNAPS) have been found in the literature, showing the scientific interest raised by this phenomenon and their putative clinical interest, especially in the field of cancer. Today, the detection of extracellular tumor-derived DNA and/or RNA is considered by many authors as a new molecular marker for situations such as cancer diagnosis, monitoring the outcome of a disease and, even, as a treatment response indicator. Furthermore, in some studies it has been suggested a possible role of tumor CNAPS in the development of metastasis. Specifically, the hypothesis known as the "genometastasis hypothesis" proposes that stem cells might be naturally transfected with dominant oncogenes as a result of dissemination of such genes in the plasma.

On the other hand, current studies concerned with the biology of metastatic cells are increasingly being focused on the striking similarities found between these cells and stem cells.

In this review we intend to expound and integrate two theories about metastatization: the "genometastasis hypothesis" and the idea of stem cells as cancer stem cells.

Key words: Cancer, Circulating nucleic acids, Stem cells, Metastasis

Introduction

The battle against cancer and the headway towards new treatments has been largely held back by the difficulties encountered in deciphering metastasis. Metastasis is the main cause of death in patients with solid tumors since the primary tumor is, in most of cases, resectable. Nevertheless, a poor understanding of the invasion and metastatic process, together with the heterogeneity displayed by cancerous cells, turns, in most cases, preferential sites of invasion to be unpredictable.

Although the steps leading to metastasis, the so called “metastatic cascade”, are well established, this pattern is still offering us a limited overview of the process which implicitly underlies a considerable amount of unanswered questions as a result of scarce knowledge on the molecular basis of cancer (Couzin, 2003).

It appears that a conceptual change is needed to understand cancer. New concepts must be found to reorganise this great puzzle.

In this review we intend to expound and integrate two theories about metastatization: the "genometastasis hypothesis" and the idea of stem cells as cancer stem cells.

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Circulating nucleic acids in plasma and serum (CNAPS) and cancer

The presence of circulating cell-free nucleic acids has been demonstrated both in disease and health. In the last decade, a burst of papers about CNAPS have been found in the literature, showing the scientific interest raised by this phenomenon and their putative clinical interest, especially in the field of cancer.

The presence of large quantities of plasma DNA in cancer patients was shown many years ago (Leon et al., 1977; Stroun et al., 1987), however, these findings did not provoke any special interest until approximately the last five years (Fig. 1). Neoplastic characteristics of plasma DNA were firstly demonstrated by Stroun et al. (1989) and, subsequently, the presence of tumor DNA in patients with diverse kinds of cancer has been repeatedly confirmed by a variety of techniques (Anker et al., 1999;

Kopreski et al., 2000; Jahr et al., 2001). Moreover, tumor-associated molecular changes, such as oncogene mutations and microsatellite alterations have been detected in plasma from cancer patients (Sorenson et al., 1994; Vasioukhin et al., 1994; Chen at al., 1996; Anker et al., 1999; Johnson and Lo, 2002). In this sense, the most studied issue in the last years has been the possible clinical value of plasmatic nucleic acids as a tumor marker (Kopreski et al., 2000; Silva et al., 2002b; Johnson and Lo, 2002; Chan and Lo, 2002; Chan et al., 2003).

Mutated ras genes were the first tumor-specific gene sequences detected in the blood from patients with cancer (Sorenson et al., 1994). Specifically, mutated k-ras sequences have particularly been attractive tumor markers in blood (Anker et al., 1997; Kopreski et al., 1997, 2000; Sorenson, 2000), since they have frequently been found in several types of commonly occurring human cancers, their presence in blood seems to be associated quite specifically with cancer and the repertoire of k-ras mutations is relatively limited, among other reasons (Sorenson, 2000).

Microsatellite alterations in plasma DNA have also been proposed as a useful tool for monitoring disease progression in cancer patients (González et al., 2000; Sozzi et al., 2001; Beau-Faller et al., 2003). However, since a panel of microsatellite markers is usually required, this analysis is still considered to be not very practical on a large scale for clinical routinary applications (Chan et al., 2003).

On the other hand, the use of Epstein-Barr virus (EBV) DNA in plasma and serum has been considered as a new tumor marker for an important group of cancers such as EBV-associated malignancies (Lo, 2000).

Another recent finding has been the detection of tumor-specific mitochondrial DNA in plasma (Jeronimo et al., 2001). As tumoral tissues contain high numbers of mitochondrial DNA sequences, detection of tumor mitochondrial DNA may offer a sensitive way to detect an early disease (Chan and Lo, 2002).

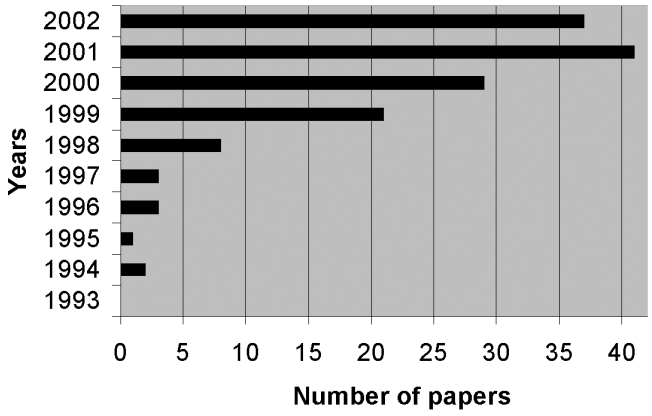

Fig. 1. Graphic representation of evolution in the amount of published studies focused on CNAPS and cancer along the last decade.

These findings become even more exciting when it was demonstrated that tumor mRNA could also be detected in plasma of cancer patients by using a similar methodology (Kopreski et al., 1999; Dasí et al., 2001; Silva et al., 2001), since it might mean that plasmatic nucleic acids are not inert molecules, but might also have a biological activity. In other words, the presence of cell-free RNA might be related to a expression profiling in plasma.

Although it has only been in the last years that the presence of tumor RNA in plasma or serum from cancer patients has raised a great interest, these findings rely on preceding studies. As long ago as 1978, Stroun et al. showed that human blood lymphocytes and frog auricles spontaneously released a nucleoprotein complex, containing RNA, in their culture medium (Stroun et al., 1978). In the 1980s, Rosi et al. demonstrated that cultured cells from human colon adenocarcinoma spontaneously released RNA-lipid complexes and suggested that this mechanism strongly resembled the shedding of viral particles from cells (Rosi et al., 1988). Moreover, they suggested that these structures might be involved in the spread of the tumor throughout the organism (Rosi et al., 1988). Simultaneously, Wieczorek et al. (1985, 1987) demonstrated that RNA-proteolipid complexes could be detected in sera of patients suffering from malignant disorders. They also suggested that these RNA-proteolipid complexes satisfied many criteria for a tumor marker (Wieczorek et al., 1987).

However, these observations have lacked continuity until the last few years, when, as mentioned before, it has been reported that tumor mRNA can be amplified from plasma or serum of patients with malignant melanoma (Kopreski et al., 1999), breast cancer (Chen et al., 2000), colon cancer (Silva et al., 2002a) and follicular lymphoma (Dasí et al., 2001), among others. Through these studies, it can be inferred that the phenomenon of circulating tumor RNA may be common to a broad range of cancers at various pathological stages.

This phenomenon was not expected, since RNase has been shown to be present in the circulation of healthy individuals and in higher concentrations in cancer patients (Reddi and Holland, 1976). In spite of the presence of such enzymes, extracellular tumor-derived RNA is not only present, but also has the sufficient integrity to permit reverse transcription-PCR (Kopreski et al., 1999). This phenomenon could be due to a protective effect of the vesicles (Ceccarini et al., 1989) or complexes (Stroun et al., 1978; Wieczorek et al., 1985; Rosi et al., 1988) in which the RNA is integrated.

Recently, Ng et al. approached this matter investigating the particle-associated nature of both circulating RNA and DNA by subjecting human plasma samples, either from cancer patients or healthy individuals, to filtration through filters with different pore sizes (Ng et al., 2002). Apart from a higher

detection of plasma mRNA in cancer patients than in healthy controls, they found that a substantial proportion of plasma mRNA species was particle-associated. However, most DNA in plasma was nonfilterable. To explain this difference, they suggested that most of the non-particle-associated RNA is degraded (Ng et al., 2002). Nevertheless, this study is in agreement with those suggesting that plasma RNA may circulate in a particle-associated form that protect it from degradation, such as within apoptotic bodies (Hasselmann et al., 2001).

Summarizing, today, the detection of extracellular tumor-derived DNA and/or RNA is considered by many authors as a new molecular marker for situations such as cancer diagnosis, monitoring the outcome of a disease and, even, as a treatment response indicator, which implies that it might be on the verge of being translated into clinical use (Kopreski et al., 1999, 2000; Chen et al., 2000; Lo, 2000; Ryan et al., 2000; Dasí et al., 2001; Silva et al., 2001, 2002a,b; Chan and Lo, 2002; Johnson and Lo, 2002; Chan et al., 2003). Many efforts are being conducted to simplify the technology to allow the use of these biomarkers for early detection of cancer in mass screenings (Wang et al., 2003). However, the origin and posterior effects of such nucleic acids remain unclear.

The enigmatic origin of plasma nucleic acids

It can be presumed that circulating DNA in healthy subjects derives from lymphocytes or other nucleated cells (Stroun et al., 2000), and there is no doubt that a substantial proportion of plasma nucleic acids derive from tumor cells, but the release mechanisms of these nucleic acids into plasma is still unknown.

As mentioned above, in the 1970's, the group of Paul Anker, Pierre Maurice, Maurice Stroun and colleagues observed that human lymphocytes spontaneously released DNA (Anker et al., 1975) and RNA (Stroun et al., 1978). They suggested that, in both cases, this release seemed to be an active mechanism not related to cell death. Later on, two groups supported the observation of RNA release, this time in human colon carcinoma cells (Rosi et al., 1988; Ceccarini et al., 1989).

Wieczorek et al. (1985) also suggested that the RNA-proteolipid complexes detected in sera of patients suffering from malignant disorders could not be merely a degradation product of cancer cells, but could also represent a specific secretory product of tumor cells, which may mediate host-tumor interactions.

Today, this issue has opened the door to controversy. It appears that the presence of tumor DNA in the blood stream could be due either to tumor cell death, whether by necrosis or apoptosis, or to a mechanism for active release. Many authors have postulated cell death as the source of plasmatic tumor DNA (Fournie et al., 1995; Jahr et al., 2001; Lichtenstein et al., 2001), however, the mechanisms have not been determined yet. Lichtenstein et al. (2001) suggested that necrosis could not be responsible for a significant part of plasma DNA, since only a small portion of DNA escapes from necrotic degradation, and, moreover, it would be difficult for necrotic DNA from cells dying in ischemic regions to appear in the blood stream. However, Jahr et al. (2001) showed, both in *vivo* and *in vitro*, that soluble DNA in the form of chromatin fragments was released from apoptotic and necrotic cells and could eventually appear in the blood stream.

On the other hand, others do not discard a spontaneous releasing of such DNA (Stroun et al., 1987, 2000, 2001; Anker et al., 1999). They firstly based this assumption on the results obtained from many *in vitro* studies, such as those mentioned above, but later, some in vivo studies supported this theory. In one of them, Stroun et al. found that the proportion of repetitive sequences (Alu sequences) compared to b-globin was significantly greater in serum DNA than in lymphocyte DNA, both in cancer patients and in healthy individuals (Stroun et al., 2001). To explain it, they suggested that there was a preferential release of repetitive sequences compared to coding genes, which argues in favor of active DNA release (Stroun et al., 2001).

Nevertheless, to date, no study has succeed in determining the relation between cell death and/or active release and the presence of plasma DNA. However, when reviewed, it appears that the more adequate conclusions might be those expound by Anker et al. "The presence of tumor DNA in the plasma is probably the result, in variable proportions, of the different mechanisms which produce leakage or excretion of DNA" (Anker et al., 1999).

The possible role of plasma nucleic acids on tumor progression and metastases development

Whatever the origin of circulating nucleic acids in plasma of cancer patients, the facts are that they are present in high amounts, protected against enzymatic action and have such integrity that permit them to be amplified. Based on these results, thinking in a functionality for such circulating nucleic acids does not seem a wild idea.

On 1999, García-Olmo et al. showed in a rat model of colon cancer that plasma of tumor-bearing rats was able to stably transform cultured cells (Fig. 2; García-Olmo et al., 1999; García-Olmo and García-Olmo, 2001) and proposed the following hypothesis: "Metastasis might occur via transfection of susceptible cells, located in distant target organs, with dominant oncogenes that are derived from the primary tumor and are circulating in the plasma". This putative phenomenon was tentatively named "genometastasis" (García-Olmo et al., 1999, 2000; García-Olmo and García-Olmo, 2001). As a possible mechanism for genometastasis they proposed that described by Holmgren et al. (1999), who demonstrated that genomic DNA from apoptotic bodies was transferred to the nuclear compartment of phagocytosing cells and that this transferred DNA was

stable over time. According to this theory, it might be possible that apoptotic bodies, derived from tumor cells and circulating in the plasma, could be taken up by phagocytosing cells and this phenomenon might be associated with the dissemination of cancer. Horizontal transfer of DNA might occur between cancer cells and other somatic cells and such transfer would provide a putative mechanism for "genometastasis" (García-Olmo et al., 2000). This hypothesis is also in agreement with the idea that plasma nucleic acids circulate protected from enzymatic degradation within apoptotic bodies or within other kinds of protective membranes. Moreover, the possibility that DNA circulates enveloped could explain the membrane affinity described in metastasis distribution long time ago (Nicolson, 1991).

Subsequently, the group of L. Holmgren (Bergsmedh et al., 2001) provided evidence that tumor DNA might be horizontally transferred by the uptake of apoptotic bodies, suggesting that lateral transfer of DNA between eukaryotic cells might result in aneuploidy and the accumulation of genetic changes that are necessary for tumor formation. Why not for metastasising?

The evidence that eukaryotic cells may be transformed by horizontal transfer of tumor DNA and the hypothesis that such transformation might be the origin of metastases give rise to a new question: Which cells would be susceptible to be transfected?

In the "genometastasis" hypothesis, the authors raised the following question: "Could normal cells, for example stem cells, that are located at a distance from a primary tumor become naturally transfected with dominant oncogenes as a result of dissemination of such genes in the plasma?" (García-Olmo et al., 1999; García-Olmo and García-Olmo, 2001).

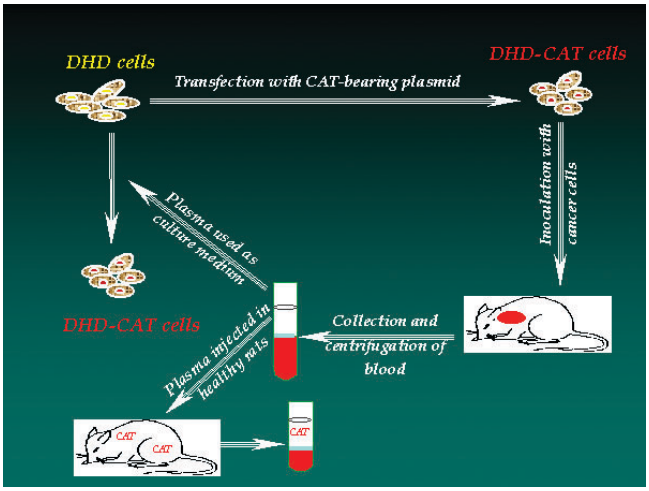

Fig. 2. Schematic representation of CAT-transfection experiments. DHD cells were converted to tagged cells (DHD-CAT cells) in two ways: as a results of direct transfection or as results of culture in medium supplemented with plasma from rats with DHD-CAT cancerous tumors. Reproduced from Histology and Histopathology (García-Olmo et al., 1999).

Stem cells have been frequently proposed as the origin of carcinogenesis (as mentioned below), thus, it would be interesting to redirect this proposal to metastasis.

Cancer, metastatic cells and stem cells

The idea of stem-cell implications in cancer was already proposed in hypothetical terms in “the stem cell theory” (Fialkow, 1979; Till, 1982; Kondo, 1983; Greaves, 1986; Trosko and Chang, 1989). According to this model, carcinogenesis would start at a stem cell which suffers genetic changes that make it unable to terminally differentiate and proliferate, rending it the ability to invade other tissues and spread throughout the body. By the revision of recent studies, S. Pathak has proposed the hypothesis that all cancers, solid as well as hematological, arise from organ- or tissue-specific stem cells, not from fully differentiated somatic cells (Pathak, 2002). However, controversy arises from results which point at fully differentiated cells as the targets for transformation (Fodde et al., 2001).

These divergent opinions on the origin of carcinogenesis become even more varied and complex when exploring the process of metastasis.

The fact that tumors are inherently heterogeneous, composed of phenotypically distinct cancer cells, led to the assumption that within the tumor bulk a reduced number of rare cells with the ability to metastasise might exist (Fidler and Kripke, 1977; Poste and Fidler, 1980).

Current studies concerned with the biology of metastatic cells are taking the process of metastasis up again, and, furthermore, interest is increasingly being focused on the striking similarities found between these “rare” cells and stem cells.

A recent model of cancer metastasis proposed by Bernards and Weinberg (Bernards and Weingberg, 2002) and largely discussed in various letters to Nature (Edwards, 2002; Gatenby and Maini, 2002; Sherley, 2002) challenged this assumption: Metastatic cells would be the result of the genetic state of the primary tumor more than of emergent “rare cells” (Bernards and Weingberg, 2002). This model, proposed in theoretical grounds, is supported by the work, now based on experimental results, of Ramaswamy et al. (2001). This new study presents a signature for metastatic potential found in cells within the primary tumor and discusses that the majority of cells in a tumor, not just rare cells, have the potential to metastasise. It is corroborated by the discovery, reported by Liotta and Kohn (2003), that different tumor types have similar pathways to metastasise, therefore making metastasis to be more easily predicted by looking at the expression profile of the primary tumor.

As these novelties are leading us to consider cancer cells as capable of acquiring metastatic potential, extensive research is also providing us with a series of evidence, which will be the conducting line of this

section, on the similarities found between metastatic and stem cells, the other group of cells capable of differentiating into various cell types.

The studies by Tu et al. (2002), documented on the work from many research groups, and Tannishtha Reya et al (Reya et al., 2002), agree on the similarities that can be found between these two, in appearance, distinct cell types:

Stem cells share with malignant cells the ability to self-renew, which renders them the ability to be immortal. At the molecular level, various studies are increasingly giving evidence that move the two cell types closer; the pathways driving self-renewal and migration of metastatic cells resemble in many ways those displayed by normal stem-cells:

- Signalling pathways regulating self-renewal of stem-cells lead to tumorgenesis when dysregulated (Sawyers et al. 1991; Sell and Pierce, 1994).

- In leukemia cells, signalling pathways, such as Notch (Screpanti et al., 2003), shh and Wnt (Taipale and Beachy, 2001) and expression of the oncogene bcl-2 (Domen and Weissman, 2000) or the recently published Bmi-1 gene regulation (Dick 2003; Lessard and Sauvageau, 2003; Park et al., 2003) are also involved in the pathways controlling stem cells self-renewal.

- A novel protein, nucleostemin, has also been found which maintains the proliferative capacity of both stem and cancer cells (Tsai and McKay, 2002).

Tu et al. take the issue further suggesting than in some ways both types of cells "seem interchangeable". They explain, based on previous studies, that the process to induce a normal stem cell from an embryo or the genital ridge to generate a carcinoma can be reverted by incorporating these carcinoma cells into the cell mass of an embryonic tissue. The cancerous cells will normalise and become part of the normal tissue (Tu et al., 2002).

While stem cells migrate to different tissues, malignant cells metastasise to different tissues. Cell separation from the matrix, migration, and settlement in a distant site to give rise to a new tissue are steps followed by both types of cells (Montell et al., 2003).

The latest studies by Montell (2003) trace parallelisms between the behaviour of epithelial cells during *D. melanogaster* development and metastatic cells, including both, the process of migration and the displayed molecular profile. The author found that signals controlling migration of border cells such as cadherins and the Jack-Stat pathway in *D. melanogaster* were also involved in breast cancer tumor invasion.

A controversial step in metastasis, being the main cause of death, is the versatility of metastic cells in "choosing" a target organ for invasion. It can be rarely predicted, leaving little room for anticipation. Invasion of stem cells in distant organs could save the step of guessing predilection since any organ would contain cells equally prone to be transformed.

Similarities between the actual processes are enlightening. As suggested by Montell (2003), cell migration in *D. melanogaster*, in which the oocyte expresses factors which attract specific cells, could be an answer to metastatic predilection for specific sites. In addition, migration of cells in clusters is also a characteristic shared by metastastatic and border cells (Montell, 2003).

Both, stem and cancer cells, have the ability to give rise to heterogeneous cell types which will also display a distinct and varied differentiation pattern. It could be due to the possible clonal origin from which both types of cells may proliferate and that could determine the potentiality of the progeny.

Stem cells divide asymmetrically, meaning that when a stem cell divides one of the daughter cells will remain stem-like while the other will start to progressively differentiate along the runs of cell division. It implies that throughout the body a reservoire composed of cells transient from pluripotent to differentiated tissue cells could be found. Moreover, recent studies have suggested that each adult tissue may have its own stem cells (Fig. 3; Temple, 2001; Jiang et al., 2002; Zuk et al., 2002).

Based on this fact Tu et al. (2002), from a personal view, proposed stem cells as the origin of metastasis and heterogeneity in solid tumors, arguing that the stage of differentiation at which the transformed stem cell is in could explain the degree of aggressiveness in metastasis and also the heterogeneity displayed by primary tumors. "Early stem cells would metastatise readily and to different organs, while late differentiated cells would metastasise rarely. Tumors not derived from stem cells would lack this ability" (Tu et al., 2002).

Following the same conducting line, a primary tumor heterogeneity would depend on the same factor, containing cell types from one or all germ layers.

The works headed by Hendrix (Hendrix et al., 2000) and Seftor (Seftor et al., 2002) provide us with a good

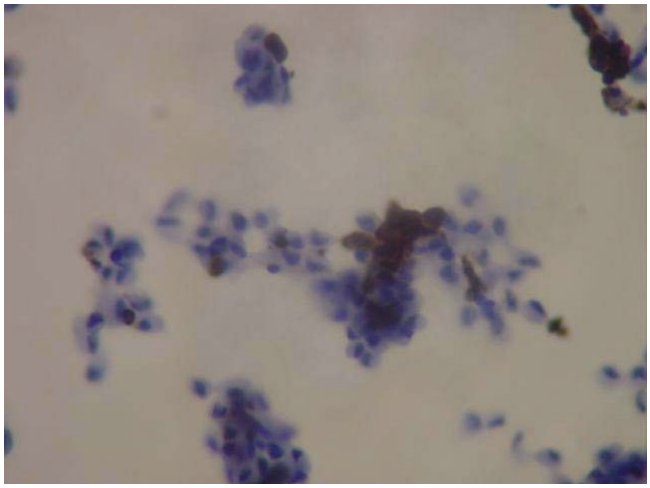

Fig. 3. Stem cells from processed lipoaspirates (García-Olmo et al., 2003) visualized by inmunohistochemical analysis using CD34 antibody. x 200

example on the versatility of metastatic cells, which, once again, conducts us to reconsider whether metastatic and stem-cells could, in fact, be interchangeable.

Some studies on aggressive breast cancer metastasis also found that aggressive cells expressed vascular markers (Hendrix et al., 1997; Thomas et al., 1999). Subsequently it was suggested that "these cells seemed to have reverted to a pluripotent embryonic-like cell capable of transformation" (Hendrix et al., 2000). A further study (Seftor et al., 2002), this time focussed on aggressive melanoma tumor cells, led them to the same conclusion. Aggressive melanoma cells displayed multiple phenotypes which resemble those of pluripotent, embryonic-like phenotyopes. Studies carried out in uveal melanoma (Folberg et al., 1993) yielded similar results.

Stem cells and cancer cells share similar telomerase activity. Telomerase is a ribonucleoprotein which adds nucleotides to the telomeres of chromosomes, protecting them from degradation, recombination and fusion at double-stranded DNA breaks. The catalytic component of telomerase in humans, hTERT, is upregulated in nearly 90% of all cancers, making it the most widely expressed marker for malignancy. With the exception of germ cells and stem cells, hTERT is undetectable in somatic human tissues. Together, these properties make telomerase a leading candidate for cancer therapy (Nguyen et al., 2003). Although telomerase activity is essential but not sufficient for neoplastic transformation, cells with such activity, as stem cells, are more likely to be transformed than cells without it, like human somatic cells (Pathak, 2002).

Comparison and integration of the results obtained from these different research groups, together with previous approaches, are close to giving a clearer picture on the possible implication of stem cells in cancer, where stem, instead of somatic cells, are starting to be considered as the origin and the targets for cancer.

All together builds a simpler and more coherent scene adding more evidence to the idea that cancer cells could appropriate the machinery for self-renewal expressed in stem cells: It seems more feasible that in the process of metastasis an immortal cell would be transformed, thus maintaining its inherent properties, than acquisition of immortality by a somatic, mortal cell, which should acquire this property de novo.

As a growing interest is increasingly being focused on stem cell biology and the similarities found between these and malignant cells, speculations on whether pluripotent cells could be the target for malignancy are rapidly emerging with promising outcomes.

Conclusion

To summarize, three facts meet to support the idea that transformation of stem cells is a putative mechanism for the origin of cancer metastasis. First of all, circulation in plasma of oncogenic cell-free nucleic acids is a widely demonstrated fact and there is evidence suggesting that such nucleic acids might be involved on metastatic process. Secondly, cancer cells and stem cells display striking similarities: both cell types self-renew, proliferate indefinitely, migrate, differentiate and express telomerase. Cancer cells differ from normal tissue-specific stem cells only in their uncontrolled growth and altered genotypes. And finally, it has been demonstrated that stem cells cultured with neoplastic plasma can change their genotype probably due to horizontal transfer of oncogenes. Then, why not consider that CNPAS might be involved in causing cancer metastasis by transformation of tissue-specific stem cells?

Indeed, a conceptual change is needed to understand and fight against cancer. We, therefore, suggest that new strategies for cancer treatment must be developed based on our current knowledge about the relationship between CNAPS, pluripotent stem cells, cancer and metastasis.

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