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Review

Altered growth pattern, not altered growth *per se*, is the hallmark of early lesions preceding cancer development

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Summary. Many human solid cancers arise from focal proliferative lesions that long precede the overt clinical appearance of the disease. The available evidence supports the notion that cancer precursor lesions are clonal in origin, and this notion forms the basis for most of the current theories on the pathogenesis of neoplastic disease. In contrast, far less attention has been devoted to the analysis of the phenotypic property that serves to define these focal lesions, i.e. their altered growth pattern. In fact, the latter is often considered a mere morphological by-product of clonal growth, with no specific relevance in the process. In the following study, evidence will be presented to support the concept that focal growth pattern is an inherent property of altered cells, independent of clonal growth; furthermore, it will be discussed how such a property, far from being merely descriptive, might indeed play a fundamental role in the sequence of events leading to the development of cancer. Within this paradigm, the earliest steps of neoplasia should be considered and analysed as defects in the mechanisms of tissue pattern formation.

Key words: Clonal growth, Focal growth, Growth pattern, Focal lesions, Tumor microenvironment

Introduction

The overt appearance of cancer, both in experimental systems and in humans, is often preceded by the presence of focal proliferative lesions (polyps, papillomas, foci, nodules, adenomas). This is not a merely temporal association; in fact it is well established that focal lesions represent a common precursor site from which cancer can arise (Foulds, 1975; Clark et al., 1984), thus implying that gaining insights into their biology and pathogenesis bears direct relevance to our understanding of the origins of neoplastic disease as a whole.

Several studies have addressed the issue regarding the possible clonal origin of cancer precursor lesions, pointing to its putative pathogenetic role (Iannacone et al., 1987; Weinberg and Iannacone, 1988; Robinson et al., 1998; Garcia et al., 2000; Diallo et al., 2001; Macaluso et al., 2003; Polyac and Hahn, 2006). In contrast, far less attention has been devoted to the analysis of the phenotypic property that serves to define these lesions, i.e. their altered growth pattern, which results in discrete collections of cells morphologically distinct from the surrounding tissue, i.e. focal lesions.

This paper discusses evidence to indicate that that focal growth pattern is a property inherent to single altered cells and is independent of clonal growth; in addition, it will be suggested how the focal nature of early precursor lesions might be of specific pathogenetic relevance in the sequence of events leading to the emergence of the overt neoplastic phenotype. In this respect, cancer should be considered a disease beginning with a defect in the mechanisms related to tissue pattern formation.

The clonal nature of early precursor lesions

It has become almost axiomatic that early lesions appearing in the course of cancer development are clonal in nature, i.e. they represent a clone of single altered cells (Iannacone et al., 1987; Weinberg and Iannacone, 1988; Robinson et al., 1998; Garcia et al., 2000). Although the foundation of this general assumption has been disputed by recent reports (Garcia et al., 2000; Diallo et al., 2001), most of the current theories on the origin of cancer are based on such a hypothesis (Garcia et al., 2000; Macaluso et al., 2003; Polyac and Hahn, 2006). A classical example is the molecular pathway proposed for pathogenesis of human colon cancer (Fearon and Volgestein, 1990). The underlying conceptual framework, derived from Knudson's two-hit

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hypothesis (Knudson, 1971), is that the pathway to cancer begins when a rare cell is hit in a critical gene regulating cell cycle and/or survival; this alteration confers on that cell the susceptibility to undergo selective clonal expansion, (with the possible contribution of microenvironmental factors, such as inflammatory cytokines). Clonal expansion per se can then set the stage for subsequent hits in other critical genes, due to continuous cell replication (and the possible contributory role of any genotoxic microenvironment, e.g. inflammation, free radicals, etc.). The end result of this process is the emergence of cells with a full malignant phenotype, including invasive growth and metastatic capacity (Hanahan and Weinberg, 2000) (Fig. 1).

Within this hypothesis, the clonal nature of early lesions is given special emphasis and is attributed a specific pathogenetic role towards neoplasia in that (i) it allows for the stepwise, cumulative acquisition of relevant genetic changes, which are progressively transmitted from the initial cell to its progeny, until the complete set of alterations appear in the cancer cell (Corson and Gallie, 2007); and (ii) it provides the driving force (continuous cell replication, with the possible contribution of a genotoxic environment) for these progressive genetic changes to occur (Cairns, 2006; Ellegren, 2007; Rando, 2007). A further extrapolation of this general scheme has also been proposed: some Authors have gone all the way to contend that clonality *per se* should be regarded as a criterion to establish, or at least suspect, the preneoplastic nature of any focal lesion (Noguchi et al., 1992; Walsh et al., 1996; Niho et al., 1999), implying that clonal expansion alone might be sufficient to drive the entire carcinogenic sequence.

The focal nature of early precursor lesions

While the clonal nature of early lesions refers to their putative origin from a single altered cell, the term *focal* is purely descriptive and is based on histological appearance of these lesions, i.e. discrete collections of cells which are sufficiently distinguishable from the surrounding tissue in terms of overall architecture and/or cytological features (Shpitz et al., 1998; Hruban et al., 2005; Libbrecht et al., 2005; Park and Roncali, 2006; Bernam et al., 2006; Costa, 2006). From the pathologist's point of view, this is the essential diagnostic feature of focal lesions, irrespective of the type of tissue or organ involved (e.g. aberrant crypt foci in the colon, foci/nodules of phenotypically altered cells in the liver, etc). As such, focal lesions can be either monoclonal or polyclonal, in that they are solely defined on morphological grounds.

It is rather surprising that the focal nature of early precancerous lesions is a relatively neglected topic, at least compared to the issue about clonality referred to above. Furthermore, the terms focal and clonal are often used interchangeably in this context (Luebeck et al., 2000); however, the emphasis is generally placed on the latter, while the former is considered a secondary by-product, which is useful for the pathologist to identify these lesions, but has no specific significance for either their origin or their fate.

In the following discussion, we would like to present evidence to suggest that focal growth pattern, far from being a merely descriptive feature, (i) is an inherent property of early precancerous lesions, (ii) is independent of clonal growth and (iii) plays a specific pathogenetic role on the pathway to neoplasia.

In this analysis, one of the first questions to consider refers to the basis of focal growth. Why do such early lesions grow as discrete collections of cells, in a pattern that is clearly different from that of surrounding tissue? This phenomenon is most evident in solid organs, where early foci progressively grow to form spheroid nodules, showing no tendency to integrate into surrounding tissue and displaying a sharp demarcation between normal and altered cell populations (Libbrecht et al., 2005). A simplistic, almost intuitive explanation for such growth pattern envisions focal growth as a consequence of clonal growth; i.e. the selective clonal expansion of a single altered cell in a solid tissue is bound to result in a focal growth pattern with the histological features

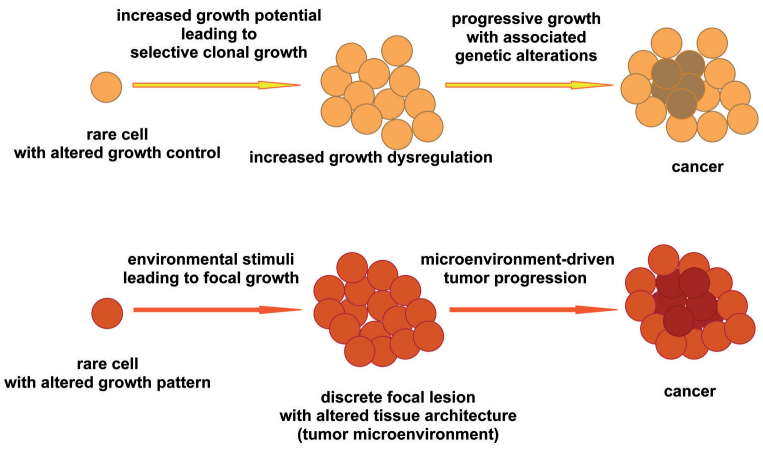

Fig. 1. Top. According to this general pathway, cancer development begins as a disease in the regulation of cellular growth control mechanisms. This results in the selective clonal expansion of the rare cells with altered genotype/phenotype, leading to further accumulation of genetic damage and increasing deregulated growth. The end result of the process is the emergence of an overt malignant neoplasm. Bottom. This alternative view of cancer development places major emphasis in the altered growth pattern of early focal lesions. According to this proposition, cancer begins as a disease of tissue pattern formation, leading to the emergence of a peculiar tumor microenvironment. The latter constitutes a new biological niche with peculiar biochemical alterations, which in turn set the stage for tumor progression.

described above. It is obvious that within this paradigm, the focal nature of early lesions becomes a simple by-product of clonal growth and, as such, is likely to have no specific pathogenetic relevance. This in turn might explain the relative paucity of studies in the literature addressing this issue.

The above argument linking focality to clonality might indeed appear rather straightforward and appealing in its simplicity; however, based on present knowledge, it is a difficult hypothesis to sustain.

The first line of evidence against this hypothesis is the simple fact that clonal growth does occur during development and throughout post-natal life, and in none of these cases is associated with focal growth, as defined above.

However, one could still argue that the clonal expansion of a rare cell in a fully differentiated (or adult) tissue might follow a pattern which is different from that observed during normal development and normal tissue turnover. Recent findings from our laboratory have conclusively ruled out this latter possibility (Laconi et al., 2001a,b). Using a rat model for cell transplantation, we analysed the growth pattern of either normal or altered/nodular hepatocytes injected into the liver of adult recipients. In order to stimulate the clonal expansion of transplanted cells, host animals were pre-treated with retrorsine, an agent which imposes a persistent mitotic block on endogenous hepatocytes (Laconi et al., 1999). In this setting, only transplanted cells, delivered after the chemical is metabolised, selectively proliferate upon appropriate stimulation. Either normal or altered hepatocytes (the latter were isolated from carcinogen-induced hepatic nodules) were then injected into retrorsine-treated animals. As predicted, both cell types were able to form clones in the recipient liver (Laconi et al., 2001a,b). However, the overall outcome, including growth pattern and biological fate, was sharply different. While transplanted normal hepatocytes integrated into the host liver, established regular junctions and formed hybrid bile canaliculi with resident parenchymal cells, nodular hepatocytes grew as discrete focal lesions, did not integrate in the surrounding liver and developed into nodules which in fact compressed the host tissue. Moreover, with time, normal hepatocyte transplantation resulted in massive repopulation of the resident liver, with seemingly normal liver histology and preserved liver function (Laconi et al., 1998, 2001a); in contrast, the final outcome of altered hepatocyte transplantation was the development of hepatocellular carcinoma originating from donor cell-derived hepatocyte nodules (Laconi et al., 2001b).

It is also pertinent to mention that neither normal nor altered hepatocytes could clonally expand following transplantation into the liver of untreated recipients, i.e. the microenvironment of a normal liver, unlike that of retrorsine-injured animals, did not stimulate the selective expansion of either cell types (Laconi et al., 1998, 2001a,b).

Taken together, these findings provide important indications relevant to the present discussion. Firstly, selective clonal expansion of isolated cells in an adult tissue is not necessarily associated with a focal growth pattern, in that normal hepatocytes transplanted into retrorsine-treated rat liver underwent extensive clonal growth, integrated into the recipient tissue and established a normal histology, with no evidence of focal lesions (Laconi et al., 1998, 2001a). Incidentally, similar results have been reported during the analysis of other available models of extensive liver repopulation following transplantation of isolated hepatocytes (Rhim et al., 1994; Overturf et al., 1996; De Vree et al., 2000). These type of findings unequivocally indicate that the focal growth pattern associated with altered/nodular cells, as also observed in our studies (Laconi et al., 2001b), cannot be explained simply on the basis of their selective clonal expansion, and alternative explanations must be considered and explored.

Secondly, the growth behaviour of transplanted normal and altered hepatocytes appears to be qualitatively similar, in that (i) both cell types could clonally expand in the retrorsine-treated host liver; however, (ii) neither cell type was able to proliferate significantly following transplantation into a normal liver (Laconi et al., 1998, 2001a,b). These latter findings are not consistent with the hypothesis that defects in growth control mechanisms represent an early hallmark of altered cells in the carcinogenic pathway (Fearon and Volgestein, 1990; Hanahan and Weinberg, 2000; Malumbres and Barbacid, 2001). Conversely, in the absence of obvious alterations in growth regulatory parameters, the focal growth pattern of altered/nodular cells emerges as the most significant difference between those cells and the clonally expanding normal hepatocyte population described in our studies (Marongiu et al., 2008).

The possible basis for the focal growth pattern

If focal growth is not simply a by-product of clonal growth, what is/are the biological and molecular determinants of such an altered pattern of cell and tissue architecture in early lesions associated with neoplastic disease? Although no direct answer to this question is yet available, several data existing in the literature do offer important insights into the topic. Of particular relevance in this context are a long series of studies describing changes in the expression of proteins involved in cell-to-cell and/or cell-to-extracellular matrix (ECM) communication during early phases of cancer development in various tissues. Almost 30 years ago Potter suggested in a comprehensive review the importance of studies on intercellular communication for the analysis of multistage carcinogenesis (Potter, 1980). The research groups of Yamasaki and colleagues (Yamasaki et al., 1984) and Trosko and colleagues (Loch-Caruso and Trosko, 1985) were among the first to present data indicating that transformed cells were relatively unable to communicate with their normal counterparts. An in vitro test was proposed to identify agents that could interfere with cell-to-cell coupling,

implying that such agents should be considered at risk for the promotion of the neoplastic phenotype in vivo (Barrett et al., 1986). In more recent years, evidence has been gathered that altered expression of components mediating cell-to-cell and/or cell-to-ECM interactions is a common finding during cancer development in virtually all tissues, both in human and experimental systems (Pugacheva et al., 2006; Mikels and Nusse, 2006; Christofori, 2006; Dalmay and Edwards, 2006; Russel and Ohh, 2007). Most importantly, some of these changes were reported to occur early in the process (Drachenberg and Papadimitriou, 1995; Valizadeh et al., 1997; Smits et al., 2000; Oliveira et al., 2006; Russel and Ohh, 2007), suggesting that they could have a role right at the initial stages of the pathogenetic sequence.

However, it should be acknowledged that the latter issue has been difficult to address so far. Namely, it has been difficult to establish whether alterations in cell-to-cell and/or cell-to-ECM communication are a cause or a consequence of focal growth. For example, early focal lesions developing during liver carcinogenesis in the rat were reported to express low levels of connexin 32 (Krutovskikh et al., 1991); however, this alteration was found to be reversibly associated with the proliferative rate of individual foci (Neveu et al., 1994). Furthermore, normal proliferating hepatocytes also express low connexin 32 protein (Kren et al., 1993). Obviously, this type of information does not allow one to conclude whether the altered expression of the membrane junctional protein is a primary phenomenon or is a mere consequence of the increased mitotic activity in focal lesions (Neveu et al., 1994). In this respect, a direct comparison of two clonally expanding cell populations, growing at similar rate, such as normal vs. altered hepatocytes in the transplantation system described above, might provide important insights into the molecular bases of their different phenotypic behaviour.

Conclusions and future perspectives

Irrespective of the precise mechanism(s) involved, it is a fact that focal lesions display a growth pattern which is different compared to that of the corresponding normal tissue. As already mentioned, this is indeed the main diagnostic criterium used by the pathologist to identify such lesions. Furthermore, such a pattern appears to be inherent to altered cells in that (i) it is independent of clonal growth per se; (ii) it is maintained upon isolation of these cells from the original lesion followed by their transplantation into a secondary host (as discussed above, Laconi et al., 2001b).

Given that focal growth stands as a very general hallmark of early lesions preceding the overt development of neoplasia, the question arises as to its possible pathogenetic significance, if any.

In order to address this question, we must begin from the appreciation that focality refers to an altered tissue organization, i.e. we must shift our attention from the individual altered cells composing nodules, papillomas or polyps, and consider their pattern of growth within these lesions (Fischer et al., 2004).

By definition, focal growth translates into altered tissue architecture with varying degrees of dysplastic change (Shpitz et al., 1998; Hruban et al., 2005; Libbrecht et al., 2005; Park and Roncali, 2006; Bernam et al., 2006; Costa, 2006). This is at variance with what one sees during normal tissue development, turnover and repair. The latter are in fact genetically programmed processes and follow a defined sequence of events, where each tissue component (e.g. epithelium, connective tissue, blood vessels) provides its specific contribution in a highly coordinated fashion. Remodelling of the regenerated or repaired tissue might also occur under specific circumstances, such as the healing of a bone fracture, and this is also a finely orchestrated series of events.

In contrast, the growth of focal lesions does not follow any of the above: it is typically irregular, non-integrated and imbalanced, resulting in a tissue morphology which is atypical for that particular organ (Park et al., 1997; Shpitz et al., 1998; Hruban et al., 2005; Libbrecht et al., 2005; Park and Roncali, 2006; Bernam et al., 2006; Costa, 2006). The end product of this process is the generation of a new biological niche, with its own internal microenvironment, which sets itself outside the genetically programmed developmental plan for that tissue. The above scenario includes the possibility that the initial defect might involve mechanisms related to cell differentiation (Buscarlet and Stifani, 2007; Gottlieb et al, 2007): however, emphasis in this context is given to the altered tissue patterning that may result from any derangement of the normal pathway of differentiation in that tissue .

One of the most relevant consequences of such altered growth pattern is that focal lesions, as they grow beyond a critical size, are susceptible to experience alterations in oxygen and nutrient supply, due to an insufficient and/or abnormal in-growth of a blood vessel network (Solt et al., 1977; Semenza, 2003; Zhong et al., 2004; Hagendoorn et al., 2006). This has been attributed in some cases to the fast growth of tumors outpacing the formation of new blood vessels (Xu et al., 2005); however, this is unlikely to be the case during the slow growth of early lesions. For example, alterations in blood supply were already present in small (<2 mm in diameter) hepatic nodules developing during experimental carcinogenesis in the rat (Solt et al., 1977). It appears reasonable to hypothesize that, under these conditions, the imbalanced growth between altered cells in the focal lesion and the blood vessels network reflects more of a basic detour from the developmental program of that particular organ, rather than a simple outpacing in the growth of one tissue type over the other. Stated otherwise, the focal nature of early lesions, because of its atypical growth pattern, carries a high risk of causing significant alterations in blood supply, which in turn may cause modifications in interstitial fluid composition, leading to hypoxia, acidosis, and a series of metabolic alterations defining what is referred to as the tumor microenvironment (Li et al., 2001; Semenza, 2003;

Zhong et al., 2004; Xu et al., 2005; Capp, 2005; Gatenby et al., 2006; Hagendoorn et al., 2006; Laconi, 2007). Several excellent reviews have described how the altered milieu of the tumor microenvironment can contribute to drive the process of tumor progression (Semenza, 2003; Bindra and Glazer, 2005; Capp, 2005; Xu et al., 2005; Anderson et al., 2006; Gatenby et al., 2006), suggesting a possible link between the focal growth pattern of precursor lesions and their potential to evolve towards overt neoplasia (Fig. 1).

In summary, focal growth emerges as a key phenotypic property of early lesions during carcinogenesis. According to this paradigm, cancer development begins as a disease of tissue patterning and organization (Schwartz and Ingber, 1994; Ingber, 2002), rather than a defect in the mechanisms of growth control. A full appreciation of these and other similar findings is likely to lead to a better understanding of the biological and molecular bases of neoplastic disease.

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