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Aging is associated with increased clonogenic potential in rat liver *in vivo*

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Summary

Cancer increases with age and often arises from the selective clonal growth of altered cells. Thus, any environment favoring clonal growth per se poses a higher risk for cancer development. Using a genetically tagged animal model, we investigated whether aging is associated with increased clonogenic potential. Groups of 4-, 12-, 18-, and 24-month-old Fischer 344 rats were infused (via the portal vein) with 2×10^6 hepatocytes isolated from a normal syngenic 2-month-old donor. Animals deficient in dipeptidyl-peptidase type IV (DPP-IV–) enzyme were used as recipients, allowing for the histochemical detection of injected DPP-IV$^+$ cells. Groups of animals were sacrificed at various times thereafter. No growth of DPP-IV$^+$ transplanted hepatocytes was present after either 2 or 6 months in the liver of rats transplanted at young age, as expected. In striking contrast, significant expansion of donor-derived cells was seen in animals transplanted at the age of 18 months: clusters comprising 7–10 DPP-IV+ hepatocytes/cross-section were present after 2 months and were markedly enlarged after 6 months (mean of 88 ± 35 cells/cluster/cross-section). These results indicate that the microenvironment of the aged liver supports the clonal expansion of transplanted normal hepatocytes. Such clonogenic environments can foster the selective growth of pre-existing altered cells, thereby increasing the overall risk for cancer development associated with aging.

Key words: aging; cancer; cell transplantation; clonal growth.

Introduction

Cancer incidence increases with age, to the point that aging is considered as the strongest risk factor for neoplastic disease in humans (Peto *et al.*, 1975; Gloeckler Ries *et al.*, 2003). However, the precise nature of this relationship is still unclear (Campisi, 2003; Balducci & Ershler, 2005). Several factors have been implicated, including the progressive accumulation of critical DNA alterations in specific target cells and a decline in the immunological surveillance (Balducci & Ershler, 2005). In addition, there is increasing evidence to suggest that the microenvironment of aged tissues and organs may play a role (DePinho, 2000; Krtolica *et al.*, 2001; Campisi, 2005). Cancer often develops from precursor lesions (such as polyps, papillomas or nodules/adenomas), which in turn result from the selective clonal expansion of rare altered cells. Thus, any condition favoring the emergence of clonal growth per se has the potential to promote early steps of cancer development, thereby increasing the overall probability of neoplastic disease.

Within this context, the present study was designed to directly investigate whether aging is associated with alterations in the tissue microenvironment, which might translate into increased clonogenic potential. Using a genetically tagged, syngenic rat model, we showed that the tissue environment of the aged liver is conducive to selective clonal growth of transplanted normal hepatocytes, while virtually no growth of transplanted cells was observed in the liver of young recipients. We suggest that a similar mechanism can foster the focal growth of pre-existing altered cells in the aged tissue, thereby increasing the risk of cancer development associated with aging.

Results

All animals easily tolerated the surgical procedure and the intraportal hepatocyte transplantation (Tx). Since this is a syngenic system, transplanted cells survived well in both young and old recipients, with no signs of immunologic response. At 2 weeks post-Tx, rare donor-derived DPPIV+ hepatocytes were discerned in the liver of all groups; they were mostly single cells or doublets, and 1-2 clusters/cross-section were present in the largest lobes, with no intergroup differences (data not presented). These findings are in line with our previous studies (Laconi *et al.*, 1998, 2001a). Results obtained in animals sacrificed at 2 months after Tx are reported in Fig. 1. Virtually no growth of transplanted cells was seen in the liver of animals in group I (Tx at 4 months of age); the mean number of cells/cluster/cross-section was < 3, with a single rare cluster comprising 10 hepatocytes. By contrast, significant clonal expansion of donor-derived cells was already observed in groups III and IV, receiving Tx at 18 and 24 months of age, respectively, and sacrificed 2 months later. In group III the mean cluster size was 7 ± 4 cells/cross-section, and the largest aggregate included up to 20 DPPIV+ cells. These values were still slightly higher in group IV: mean cluster size was 10 ± 6 cells/cross-section and the largest clone comprised up to 30 donor-derived cells.

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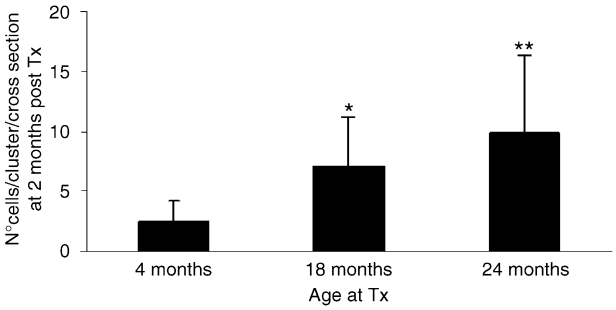

Fig. 1 Size of donor-derived hepatocyte clusters in animals from groups I, III and IV, transplanted at 4, 18 and 24 months of age, respectively, and sacrificed 2 months later. Data are mean ± SD. Significantly different from 4-month group: $*P < 0.001$; $**P < 0.0005$.

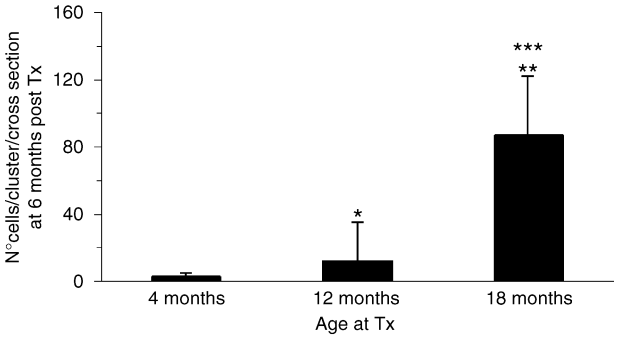

Fig. 2 Size of donor-derived hepatocyte clusters in animals from groups I, II and III, transplanted at 4, 12 and 18 months of age, respectively, and sacrificed 6 months later. Data are mean ± SD. Significantly different from 4-month group: $*P < 0.001$; $**P < 0.0001$. Significantly different from 12-month group: $***P < 0.0001$.

However, intergroup differences became prominent in rats killed at 6 months after Tx (Figs 2 and 3). Again, only small groups of DPPIV+ cells were present in the liver of animals transplanted at young age (Fig. 3a); the mean number was 3 ± 1 cells/cluster/cross-section, with < 10 cells being detected in the largest cluster. Such values were not significantly different when compared to results observed at 2 months post-Tx (see Fig. 1 vs. Fig. 2) and are in agreement with earlier studies (Laconi *et al*., 1998, 2001a). On the other hand, limited but significant proliferation of transplanted cells occurred in animals from group II, i.e. receiving Tx at 12 months of age, and sacrificed 6 months later (Figs 2 and 3b). Mean cluster size was 15 ± 17 cells/cross-section, with the largest cluster comprising up to 90 hepatocytes. But the most marked differences were found when animals from group III (Tx at 18 months of age) were analyzed at 6 months post-Tx (Figs 2 and 3c,d). Mean number of DPPIV+ cells/cluster/cross-section was nearly 90 (88 ± 35) and as many as 260 donor-derived hepatocytes/cross-section were scored in the largest clone in this group of animals (Fig. 3d).

Figures 4 and 5 report the class size distribution of donor-derived hepatocytes in different experimental groups at 2 and 6 months post-Tx. The shift towards higher class size in old animals is readily apparent. The density of DPPIV+ clusters was evaluated in animals from groups I, II and III killed at 6 months post-Tx and was related to cluster size, as expected; at < 1 cluster cm^{-2} in group I, it increased to 6–7 cm^{-2} in group II and rose to 25–30 cm^{-2} in group III.

Standard histological analysis revealed a normal liver structure in all animals from all groups. Some rats from groups II and III killed at 6 months post-Tx displayed fat accumulation in the form of large droplets, which were more pronounced in group III (Fig. 3b–d). Scattered areas of bile duct proliferation were also present in animals from group III. Hepatocytes in DPPIV+ clusters were arranged in regular trabeculae and there were no signs of compression in the surrounding host tissue (Fig. 3c,d).

Discussion

The results of our studies indicate that aging induces alterations in the liver microenvironment, which are able to sustain the clonal expansion of transplanted normal hepatocytes. Large clusters of donor-derived DPPIV+ cells (mean of 88 ± 35 and up to 260 cells/cluster/cross-section) were in fact found in the liver of recipient normal animals transplanted at 18 months of age and sacrificed 6 months later. If one approximates clusters to spheres of roughly similar size, a simple computation of average number of cells per total cluster volume, based on their mean cross-sectional size (Weibel, 1979), gives a number of about 1250 hepatocytes. This would represent a minimum of 10–11 cell cycles over a 6-month period. Such high figures were sharply contrasted by results observed in young recipients. There was virtually no selective growth (2–3 cells/cluster/cross-section) of donor-derived cells when Tx was performed at young age (4 months) and animals were similarly sacrificed after 6 months. This result came as no surprise in that it confirms our earlier findings (Laconi *et al*., 1998, 2001a) and is in line with previous data published in the literature (Gupta *et al*., 1991). A limited growth of DPPIV+ hepatocyte clusters (15 cells/cluster/cross-section) occurred in recipient rats of intermediate age (12-month-old at the time of transplantation), suggesting that this age group behaves more like young rats. Data obtained in animals transplanted at the age of 24 months and sacrificed 2 months later indicate that the trend was similar to that observed in the 18-month group, i.e. initial expansion of DPPIV+ clusters was evident (Fig. 1).

It is almost axiomatic that aging results in the progressive decline of functional and/or proliferative potential in all tissues, including liver (Bucher *et al*., 1964; Morrison *et al*., 1996; Smith & Pereira, 1996; Faragher & Kipling, 1998; Iakova *et al*., 2003; Antebi, 2005; Herbig *et al*., 2006). The analysis of recently developed transplantation systems has convincingly demonstrated that, when the growth capacity of endogenous hepatocytes is impaired, transplanted normal cells can selectively expand in the form of clonal clusters (Grompe *et al*., 1999; Laconi, 2000; Laconi & Laconi, 2002). Based on this evidence, we interpret the growth advantage of transplanted hepatocytes in the liver of aged animals as a reflection of a decreased proliferative potential of

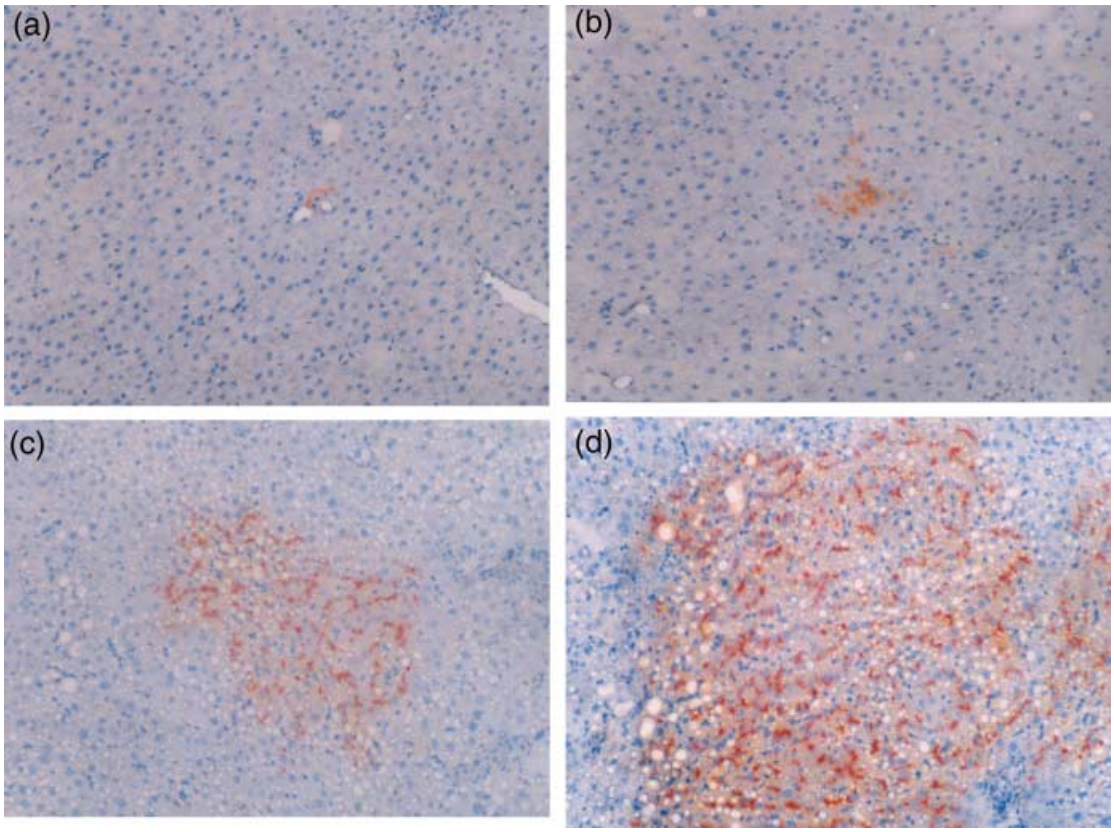

Fig. 3 Histochemical staining for dipeptidyl-peptidase type IV enzyme activity in liver sections from rats transplanted at 4, 12 and 18 months of age (groups I, II and III, respectively) and sacrificed 6 months later. (a) Isolated donor-derived hepatocytes in the liver of a young (4-month-old) recipient. (b) Small DPPIV+ hepatocyte cluster in the liver of a rat receiving transplantation at 12 months of age. (c) A large collection of DPPIV-expressing hepatocytes in the liver of a rat transplanted at 18 months of age and sacrificed after 6 months; the largest cluster in this group (d) comprised about 270 cells in cross-section. Note the presence of scattered (b) and more diffuse (c, d) fat droplets in hepatocytes from older age groups. Magnification 100x.

host parenchymal cells. In fact, in the present investigation, we found a marked decrease in the response to partial hepatectomy in aged rats compared with young controls (data not presented), in agreement with results reported in the literature (Bucher *et al*., 1964; Iakova *et al*., 2003). It is pertinent to note that a similar setting might occur in the human cirrhotic liver. Cirrhosis is associated with a reduced regenerative capacity of the liver (Chijiiwa *et al*., 1994; Marshall *et al*., 2005) and is also characterized by the presence of regenerative nodules, a fraction of which are clonal in origin (Aihara *et al*., 1994).

The observed clonal expansion of transplanted normal cells occurring in old animals suggests a novel mechanistic link between aging and carcinogenesis. Selective clonal growth of altered cells is in fact an early step of cancer development in several organs, both in humans and in experimental systems. Thus, any condition associated with increased clonogenic potential could contribute to the emergence of focal proliferative lesions originating from pre-existing altered cells, thereby increasing the risk for neoplastic disease. Several reports have described the common occurrence of putative initiated/altered cells in tissues of normal individuals (Vineis, 2003, and references therein), implying that a rate-limiting step for their selective growth is in fact the presence of an appropriate clonogenic environment. Moreover, there is evidence to suggest that the proliferative potential of altered cells does not decrease with age, in contrast to that of normal cells (Ogawa *et al*., 1985). Interestingly, a growth-constrained host environment, sustaining the clonal expansion of transplanted normal hepatocytes (Laconi *et al*., 1998, 2001a), is also able to support the growth of transplanted altered hepatocytes and their progression to cancer (Laconi *et al*., 2001b). It is conceivable that a similar mechanism can contribute to the emergence of pre-existing altered cells in the growth-impaired microenvironment of an aged tissue (Laconi *et al*., 2000; Rubin, 2001; Vineis, 2003).

Recently, senescent human prostate fibroblasts were reported to secrete factors, which appear to stimulate the growth of prostate epithelial cells *in vitro* (Bavik *et al*., 2006). The authors suggested that this may contribute to progression of prostate neoplasia in aged individuals. It is possible that similar paracrine mechanisms may also operate *in vivo* and sustain the clonal expansion of transplanted normal hepatocytes in the liver of aged rats, as observed in our studies.

In conclusion, this study indicates that aging is associated with an increased clonogenic potential in the liver *in vivo*. These results highlight a novel facet in the complex relationship between aging, clonal growth and cancer development.

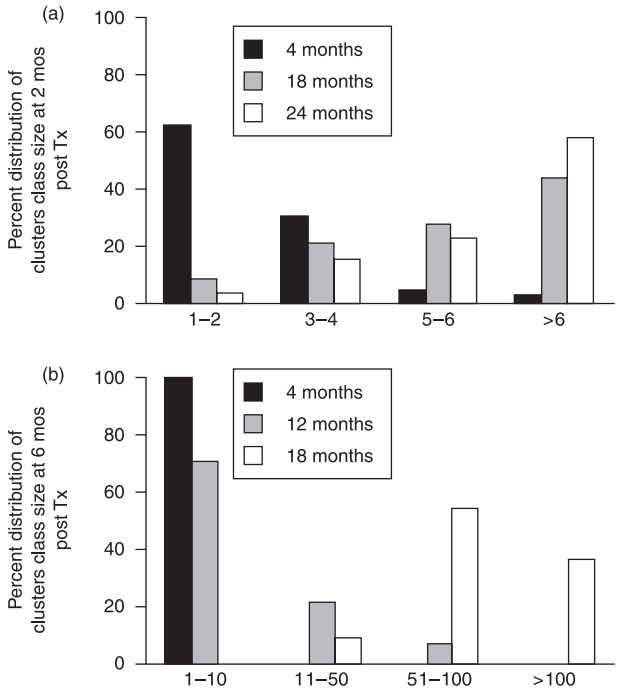

Fig. 4 Class size distribution of donor-derived hepatocyte clusters in animals transplanted at different ages and sacrificed after 2 (a) or 6 months (b). Clusters were pooled in different class sizes and expressed as a percentage of the total number of clusters in each age group.

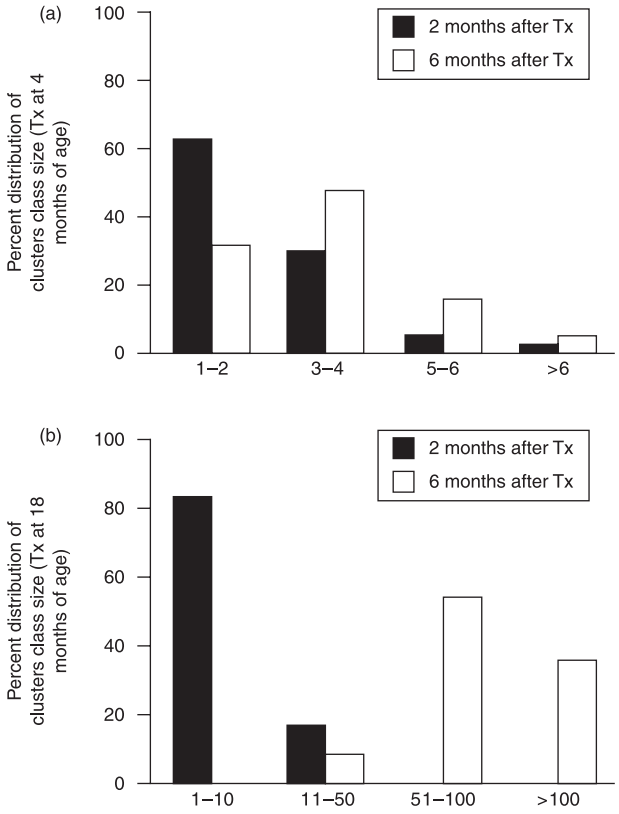

Fig. 5 Class size distribution of donor-derived hepatocyte clusters at 2 and 6 months post-Tx in animals transplanted at 4 months (a) or 18 months of age (b). Note the prominent shift towards larger class sizes in (b) between 2 and 6 post-Tx.

Experimental procedures

Animals and treatments

All animals were maintained on daily cycles of alternating 12 h light/darkness with food and water available *ad libitum*. They were fed Purina Rodent Laboratory Chow diet (Ditta Piccioni, Brescia, Italy) throughout the experiments and received humane care according to the criteria of the Animal Care Unit of the University of Cagliari. Groups of male Fischer 344 rats of 4, 12, 18 and 24 months of age (groups I, II, III and IV, respectively) were injected with 2×10^6 normal hepatocytes via portal vein infusion. Hepatocytes were isolated from a normal young adult syngenic F344 donor rat according to a standard two step collagenase perfusion technique (Seglen, 1976). The isolated cell fraction used for transplantation studies was judged to be ~95% hepatocytes by morphologic analysis and cell viability was consistently > 85%, as determined by trypan blue dye exclusion. In order to distinguish donor-derived from recipient cells in the liver, the dipeptidyl-peptidase type IV-deficient (DPPIV-) rat model was used (Thompson *et al*., 1991). A colony of DPPIV– F344 rats has been established in our laboratory, at the Department of Biomedical Sciences and Biotechnology, University of Cagliari. Such DPPIV– animals were used as recipients, while donor F344 rats were DPPIV+ and were purchased from Charles River, Milan, Italy. Three to six animals from various groups were sacrificed at different time points during the experiment, as indicated in the Results section. Liver samples were fixed in 10% buffered formaldehyde or snap frozen. Histochemical determination of DPPIV enzyme activity was performed as described (Laconi *et al*., 1998). At least 10 random sections were cut from each liver lobe of each animal and stained for DPPIV enzyme activity. Sections were then analyzed under the microscope and the number of hepatocytes for each DPPIV+ cluster was evaluated. The experiment was repeated at least twice, with different hepatocyte preparations, for each age group of animals. Data represent mean ± SD. Statistical analysis was performed using the Student's *t*-test.

Acknowledgments

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