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Psychosocial Stress Augments Tumor Development through β-Adrenergic Activation in Mice

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Housing conditions affect behavioral and biological responses of animals. We investigated the effect of same-sex-grouped (G), crowded (GC) and isolated (I) conditions on the growth of B16 melanoma or Meth A fibrosarcoma implanted in the footpad of syngeneic male C57BL/6 or BALB/c mice. Differential housing altered host resistance to tumor growth. The host responses to stress were reflected in thymic atrophy, which was lowest in the G mice, highest in the GC mice and intermediate in the I mice. The GC condition was a more stressful social environment than the I condition in both male C57BL/6 and BALB/c mice. Reflecting the extent of psychosocial stress, tumor growth was augmented in the order of GC, I and G condition, and a negative mass correlation between tumor and thymus was observed, thus clearly indicating that the host resistance to tumors was attenuated by psychosocial stress. Furthermore, the stress-enhanced tumor growth and thymus atrophy were completely abrogated by the oral administration of the non-selective β-adrenergic antagonist, propranolol. On the contrary, the chronic administration of corticosterone significantly induced the atrophy of thymus and spleen without affecting tumor growth. These results suggest an interrelationship among psychosocial stress, tumor growth and β-adrenergic activation.

Key words: Psycho-oncology — Mouse — Psychosocial stress — Tumor development — β-Adrenergic activation

Recent studies provide growing evidence for the importance of psychosocial factors in a wide variety of diseases, such as depression, cardiovascular diseases and cancer.[1–3] It has been reported that social interaction is associated with the risk of cancer incidence and mortality in humans,[4–6] although the mechanism of the association between psychosocial factors and cancer development is not yet clear. In an effort to investigate the effect of psychological stress on tumor progression, several stress paradigms have been applied to laboratory animals, including restraint,[7] rotation[8] and social housing condition.[9–11] In socially organized mammals the predominating stressors are not physical events, but arise from the immediate social environment of the animal. Therefore, social housing condition is viewed as a more natural and convenient model of psychosocial stress, and should be useful for investigating the modulatory role of psychosocial stress in tumor development.

Isolation and crowding typically evoke social stress reactions with prominent psychosocial components mimicking emotional state alterations. Social isolation (i.e. individual housing of animals) is well studied as a model lacking social interactions among animals. This model is considered to be relatively comparable with the situation of humans who feel isolated. Crowding is the situation in which large numbers of animals, including man, are restricted in environmental space. Such an occurrence may start with just a few animals. Social stress of crowding has been much less studied than isolation stress. Results from the study of population density of animals per cage suggest that crowding is a stressful factor for animals,[12, 13] pointing to a role of the adrenal glands. Studies supporting this concept have been largely based on the changes of adrenal weight and corticosterone concentration.[14–16] Although to our knowledge, studies using mammals have not yet reported the impact of crowding stress on tumor development, they have provided evidence of crowding stress-induced immunosuppression.[17, 18] Thus, the present study attempts to determine the impact of social crowding on tumor development in comparison with social isolation, and to understand the underlying mechanisms, using B16 melanoma and Meth A fibrosarcoma in syngeneic male C57BL/6 and BALB/c mice, respectively. Exposure to virtually any stressor activates the hypothalamic-pituitary-adrenal axis and results in a readily discernible elevation in plasma catecholamine and glucocorticoid levels as one of the principal adaptive responses to stress. Therefore, in the course of this study, we also studied the effects of exogenous administrations of β-adrenergic antagonist and corticosterone in male C57BL/6 mice.

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MATERIALS AND METHODS

Animals and housing Five-week-old male C57BL/6 and BALB/c mice were used in this study. These animals were obtained from Japan SLC, Inc. (Hamamatsu) and randomly assigned to be grouped, crowded or individually housed using cages with dimensions of 28.5×45×18 cm or 15×23×12 cm in the Animal Experimental Laboratory, Itto Institute of Life Science Research. The animal laboratory was maintained at constant temperature (23–25°C) and relative humidity (65%), and with a 12-h light/dark cycle under conventional conditions in accordance with the institute's animal care guidelines. There were walls and a 12 cm distance between cages for the individual housing. Food (MM-3, Funabashi Farm Co., Ltd., Funabashi) and water were available *ad libitum*. In the first experiment (experiment 1), 32 C57BL/6 mice were randomly distributed by weight into 4 groups (8 animals/group). Two sets of animals were grouped (G) or isolated (I), keeping almost the same space for each animal to live (321 or 345 cm^2/animal). In the other 2 sets, animals were reared under crowded (GC) condition (86 cm^2/animal) with or without treatment of propranolol (PPL, Sigma Chemical Co., St. Louis, MO), a non-selective β-adrenoceptor antagonist. Under group housing conditions, 4 animals were reared per cage, because the population density of 4 mice per cage is shown to induce minimal stress compared to that induced by the population densities of 2 or 8 mice per cage.[16] In the second experiment (experiment 2), 12 C57BL/6 or BALB/c mice were distributed by weight into 2 groups (6 animals/group). Using cages with dimension of 15×23×12 cm, animals were isolated (I) (345 cm^2/animal) or reared under a crowded (GC) condition (58 cm^2/animal). In the third experiment (experiment 3), 18 C57BL/6 mice were distributed by weight into 3 groups (6 animals/group). Using cages with dimension of 28.5×45×18 cm, animals were reared under a grouped (G) condition (214 cm^2/animal) with or without treatment of corticosterone (CR, Sigma), the endogenous glucocorticoid in mice. In each experiment, animals were acclimated to the housing conditions for 3 weeks before tumor implantation.

Tumors Murine transplantable cell lines, B16 and Meth A, were kindly provided by the Cell Resource Center for Biomedical Research, Institute of Development, Aging, and Cancer, Tohoku University (Sendai). The former is a melanoma that arose spontaneously in a C57BL/6 mouse and the latter is a methylcholanthrene-induced fibrosarcoma in a BALB/c mouse. Tumor cells were maintained as monolayer cultures in RPMI 1640 medium (Nissui Pharmaceutical Co., Ltd., Tokyo) supplemented with 10% fetal bovine serum (CSL, Ltd., Parkville, VIC, Australia), 100 μg/ml streptomycin, 60 μg/ml kanamycin and 100 U/ml penicillin (growth medium).

Assay for tumor growth Log-phase cultures of B16 or Meth A cells were harvested with 0.25% trypsin in Hank's balanced salt solution (HBSS) without Ca^{2+} or Mg^{2+}, washed with serum-free RPMI 1640 and resuspended at the concentration of $0.5–2\times10^7$ cells/ml in HBSS. After 3-week acclimation of animals to the housing conditions, C57BL/6 or BALB/c mice were injected subcutaneously (s.c.) with the suspension (50 μl) of B16 (experiment 1, 4×10^5/mouse; experiments 2 and 3, 2×10^5/mouse) or Meth A cells (experiment 2, 1×10^6/mouse) in the hind right footpad. Tumor growth was measured every 2–4 days and expressed as volume, which was calculated using the following formula: tumor volume (mm^3)=0.4×(large diameter)×(small diameter)^2.[19] Animals were sacrificed at the indicated time after tumor implantation to weigh the tumors and various organs.

Drug administration Mice were given the drinking water dosed with 30 ppm of PPL from day −21 to day 21 of tumor implantation or 30–60 ppm of CR from day 0 onwards. Dosage was calculated from the measurements of water intake and animal body weight.

Statistical analysis Student's two-sided *t* test was used to examine the significance of differences among groups.

RESULTS

Effects of differential housing conditions on the growth of B16 melanoma cells With respect to housing conditions, at least 2 factors are believed to evoke social stress reactions. One is the living space per animal. The other is the population density of animals per cage. We first examined the effects of housing population density of animals on the growth of B16 melanoma implanted in male C57BL/6 mice, which were grouped (G, 321 cm^2/animal), isolated (I, 345 cm^2/animal) or reared under a crowded (GC, 86 cm^2/animal) condition with or without PPL treatment (experiment 1). Fig. 1 shows that tumor growth was influenced by social housing conditions. A marked enhancement of tumor growth, i.e. increase of tumor size, was observed in the order of GC, I, and G housing. Interestingly, the treatment of GC mice with PPL reduced initial rate of tumor growth more strongly than that seen in G mice. The difference in tumor size between animals under these housing conditions, however, became smaller with the passage of time after tumor inoculation.

Organ weight of the mice reared under differential housing conditions In experiment 1, there were no discernible differences of body weight between the tumor-bearing mice reared under differential housing conditions (data not shown). Because stress is well known to induce adrenal hypertrophy and lymphoid tissue atrophy, the animals were sacrificed on day 21 after tumor implantation to weigh the adrenal and lymphoid organs. As shown in Fig. 2, adrenal and spleen weight tended to increase, thymus

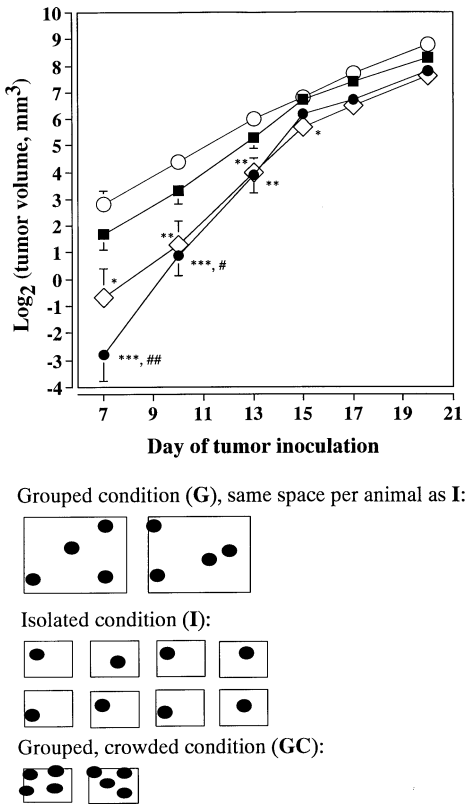

Fig. 1. Effect of different housing of mice on the growth of B16 melanoma. Eight male C57BL/6 mice were differentially housed for 3 weeks using cages with dimensions of 28.5×45×18 cm (group, G ◇) or 15×23×12 cm (isolation, I ■; crowding, GC ○; or PPL-treated crowding, PPL ●), after which B16 cells (4×10^5/mouse) were implanted s.c. in the hind right footpad (experiment 1). Tumor size was measured every 2–4 days and is expressed as tumor volume. Each point represents the mean of tumor volume. Bar, SEM. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ vs. GC; #, $P<0.05$; ##, $P<0.01$ vs. I, by Student's two-sided *t* test.

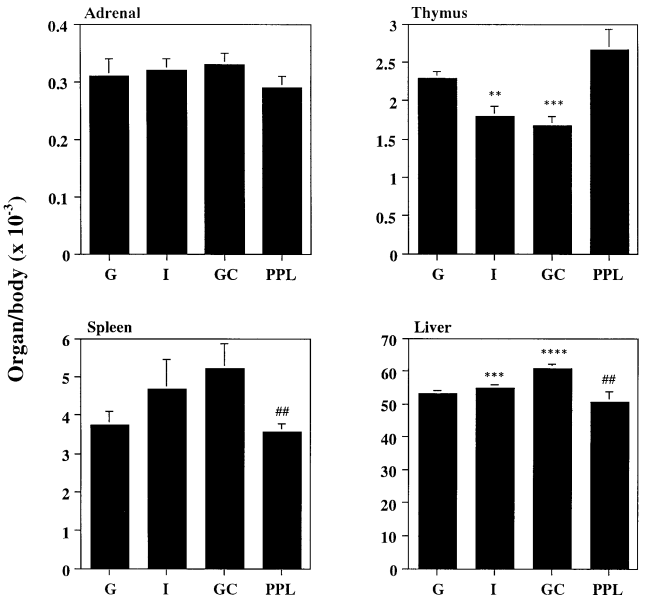

Fig. 2. Effect of different housing of mice on the lymphoid organs. In experiment 1, the tumor-bearing mice were sacrificed on day 21 after tumor implantation, and the adrenal, thymus, spleen and liver were weighed (G ◇, I ■, GC ○: see Fig. 1). Each column represents the mean of organ/body weight. Bar, SEM. **, $P<0.02$; ***, $P<0.01$; ****, $P<0.001$ vs. G; ##, $P<0.02$ vs. GC, by Student's two-sided *t* test.

weight was significantly decreased, and liver weight was significantly increased in I and GC mice compared with G mice. The adrenal hypertrophy and thymic atrophy indicates that differential housing conditions indeed constituted a significant psychosocial stress for male C57BL/6 mice: the stress responses seem lowest in G mice, highest in GC mice, and intermediate in I mice. In addition, the physiological responses induced in GC mice were completely abrogated by PPL treatment, to the same levels as seen in G mice (Fig. 2). The mass correlation of tumor with lymphoid organs was analyzed in all the mice except for PPL-treated GC mice. Tumor mass was negatively correlated with thymus mass ($P<0.05$), but positively with spleen mass ($P<0.001$) and liver mass ($P<0.01$) (Fig. 3). The negative mass correlation between tumor and thymus shows that resistance to tumor growth was positively correlated with thymus weight. In contrast, the positive mass correlation between tumor and spleen or liver suggests organ hypertrophy resulted from tumor growth.

Comparison of tumor growth between crowded and isolated conditions To clarify whether or not the GC condition is more stressful than the I condition, we further investigated the effect of GC and I housing on tumor growth, using B16 melanoma and Meth A fibrosarcoma (experiment 2). The employed GC condition (58 cm^2/animal) was not overcrowding but rather normal for mice compared to the experimental condition used by Csermely *et al.* (normal, 68 cm^2/animal; overcrowding, 22 cm^2/animal).[18] Because the host response to stress was reflected in thymic atrophy (Fig. 2), the mass correlation between tumor and thymus was analyzed in the tumor-bearing C57BL/6 or BALB/c mice reared under crowded or isolated conditions. As shown in Fig. 4, both C57BL/6 and BALB/c mice showed a negative correlation ($P<0.05$) between tumor mass and thymus mass. In addition, the GC condition gave rise to a significant increase of tumor weight and a decrease of thymus weight compared to the I condition. The effects of the GC condition

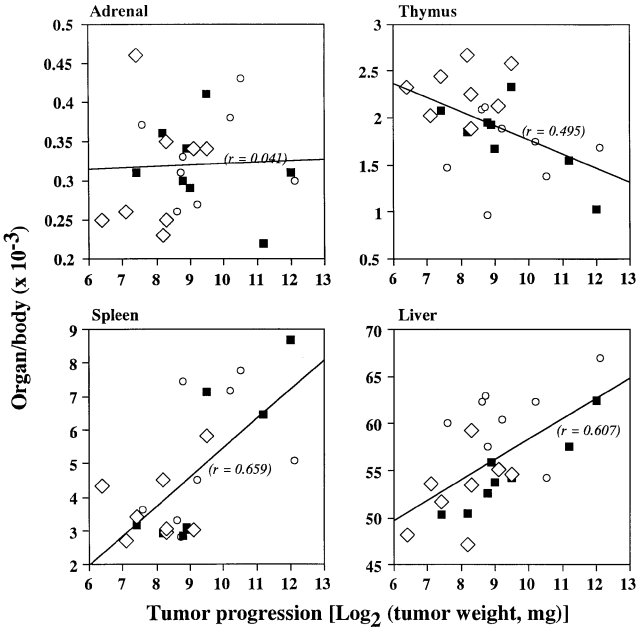

Fig. 3. Mass correlation between tumor and lymphoid organs (G ◇, I ■, GC ○: see Fig. 1).

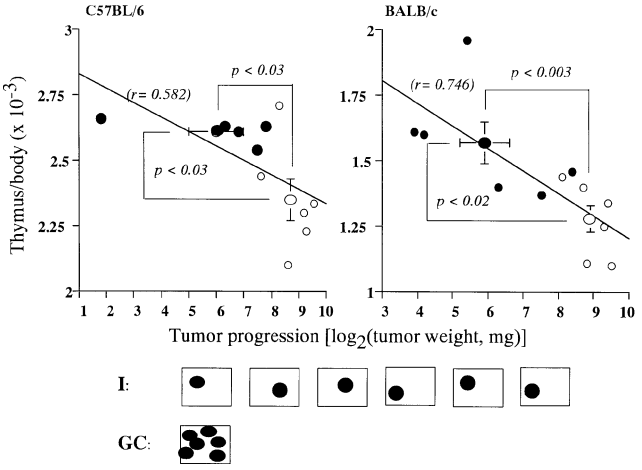

Fig. 4. Effect of I and GC housings in mice on tumor growth and thymus atrophy. Six male C57BL/6 or BALB/c mice per group were housed under an isolated (I, ●) or a crowded (GC, ○) condition for 3 weeks using cages with dimensions of 15×23×12 cm, after which tumor cells (2×10^5/mouse B16 melanoma for C57BL/6; 1×10^6/mouse Meth A fibrosarcoma for BALB/c) were implanted s.c. in the hind right footpad (experiment 2). The tumor and thymus were weighed 14 days following tumor inoculation. The larger symbols represent the mean of 6 mice per each group. Bar, SEM. Statistical significance of differences among groups was evaluated by using Student's two-sided *t* test.

on tumor growth and thymic atrophy were greater in BALB/c mice than in C57BL/6 mice, suggesting that BALB/c mice are more susceptible to psychosocial stress than C57BL/6 mice.

CR effects on tumor growth and lymphoid atrophy Interaction between the immune and endocrine systems was first noted in the early work of Selye,[20] showing that stress-induced lymphoid tissue atrophy is mediated by the adrenal gland. Glucocorticoids, released from the adrenal cortex, were subsequently identified as the hormonal agents responsible for this lymphoid atrophy.[21] To investigate the effects of CR on tumor growth and lymphoid atrophy, physiological doses of CR were exogenously administered to male C57BL/6 mice implanted with B16 melanoma (experiment 3). CR administration resulted in dose-dependent atrophy of the thymus and spleen without affecting tumor growth (Fig. 5, A and B). The CR-derived thymic atrophy, restricting the negative mass correlation between tumor and thymus (Fig. 5C), indicates that elevated CR levels in the plasma are involved in thymic atrophy, but not tumor growth.

DISCUSSION

Differential housing of experimental animals is a relatively naturalistic social condition compared to such stress paradigms as restraint, rotation and forced swimming. Despite the usage of genetically similar mouse colonies and tumor cell lines, the mice reared under differential housing conditions showed differential tumor growth (Fig. 1). The host responses to stress were reflected in thymic atrophy (Fig. 2). The negative mass correlation between tumor and thymus (Figs. 3 and 4) clearly indicates that the host resistance to tumors is attenuated by differential housing-induced stress. This finding suggests that social interaction, i.e. psychosocial stress, affects tumor development.

Fighting behavior was observed among crowded mice more frequently than among grouped mice (data not shown). Fighting between mice is involved in the establishment of dominance hierarchies.[16] Social hierarchy development is shown to play an important role in animal stress.[16] Reflecting the ethological nature of mice, a grouped condition induced minimal stress compared to either an isolated or a crowded condition. When the degree of stress was compared between crowding and isolation, crowding was shown to be a more stressful condition than isolation in both C57BL/6 and BALB/c mouse strains (Fig. 4). This result was similar to that seen in rats with respect to sensitivity to restraint ulcers.[21] We have previously shown that social isolation stress suppresses the basal immune responses of male BALB/c mice, including a reduction of splenic NK activity,[10] and also accelerates

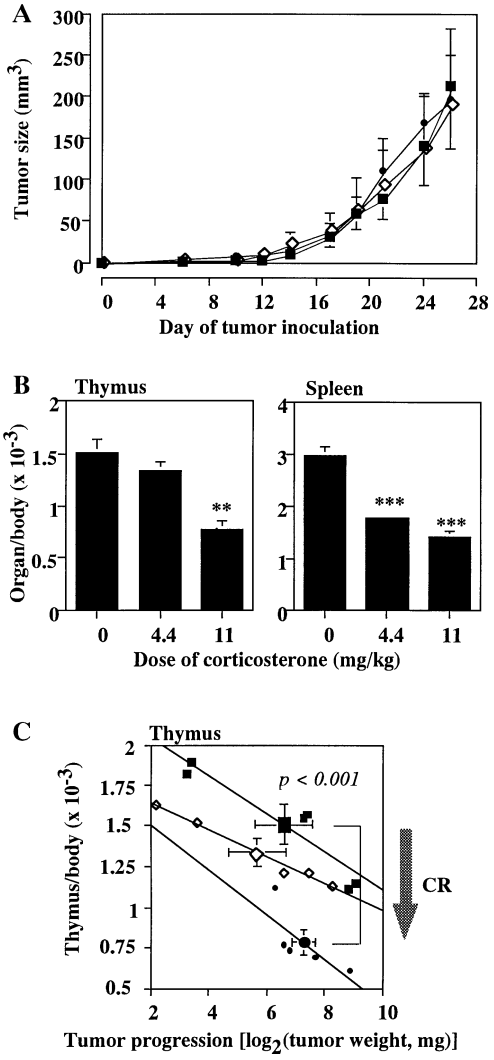

Fig. 5. Effect of administration of CR on tumor growth and lymphoid atrophy. Five to six male C57BL/6 mice per group were reared under the grouped condition for 3 weeks using cages with dimensions of 28.5×45×18 cm, after which tumor cells (2×10^5/mouse) were implanted s.c. in the hind right footpad. CR was administered from day 0 of tumor inoculation onwards (■, untreated; ◇, 4.4 mg/kg; ●, 11 mg/kg) (experiment 3). A, Tumor size was measured every 2–3 days and expressed as volume. Each point represents the mean of 5–6 animals. Bar, SEM. B, The thymus and spleen were weighed 26 days following tumor inoculation. C, For analysis of mass correlation between tumor and thymus, the value of thymus/body weight ratio was plotted against that of tumor weight.

tumor-induced angiogenesis, probably by up-regulating angiogenesis-related factors such as tumor necrosis factor-α, vascular endothelial growth factor and hepatocyte growth factor.[22] The complete abolition of stress-augmented tumor growth by a β-adrenergic antagonist (Fig. 1) suggests a substantial adrenergic involvement in the crowding-induced alteration of tumor growth.

With respect to the mechanism underlying crowding-augmented tumor growth, there is abundant indirect evidence of sympathetic nervous system involvement in the suppression of NK activity: adrenaline suppresses NK activity via β-adrenoceptor activation followed by down-regulation of perforin secretion.[23–25] Thus, the abolition of enhanced tumor growth by PPL (Fig. 1) suggests a reversal effect on suppressed NK activity via β-receptor blockade. Because adrenergic activation also increases the population of not only stress-sensitive NK cells, but also NKT cells that are resistant to stress,[26] the time-dependent reduction of the difference in tumor growth under differential housing conditions (Fig. 1) and the tumor-dependent splenomegaly and hepatomegaly (Fig. 3) imply that other immune cells including NKT cells participate in the host defense against tumors.

The administration of CR indeed caused dose-dependent lymphoid atrophy, but hardly altered tumor growth (Fig. 5). Glucocorticoids are known to induce programmed cell death with the morphologic characteristics of apoptosis in cells of the lymphoid lineage at certain stages of differentiation by activating cytosolic glucocorticoid receptors and inducing caspase-3 activity.[27, 28] However, positive autoregulation is a necessary component of glucocorticoid-induced apoptosis in glucocorticoid-sensitive T cells.[29] Therefore, CR-sensitive lymphoid cells seem to be little involved in tumor cell destruction in this tumor model. Moreover, plasma CR does not appear to be a major *in vivo* regulator of NK activity. Since the dosage of 11 mg/kg CR significantly increased food intake and body weight gain compared to the untreated mice, though such change in body weight gain was not observed in the I and GC mice (data not shown), the CR dosage appears to induce a higher plasma level than the endogenous CR level elevated by exposure to crowding and isolation stress. On the other hand, the treatment of stress-exposed mice with PPL produced a remarkable retardation of tumor progression (Fig. 1), together with a tendency to improve stress-induced adrenal hypertrophy and thymic atrophy (Fig. 2). Although there is a little evidence for noradrenergic involvement in thymic atrophy,[30, 31] the mechanism underlying the suppressive effect of a β-blocker on these organs remains to be examined in detail. Therefore, the negative mass correlation between tumor and thymus (Figs. 3 and 4) may be associated with enhancement of tumor growth by adrenaline-induced suppression of NK activity and CR- or/and adrenaline-derived thymic atrophy. To reverse suppressed NK activity, a β-blocker, if used at a dosage that would not induce hypotension, might be available for cancer patients in a psychosocially stressful environment.

We have demonstrated here the interrelationship among psychosocial stress, tumor development and β-adrenergic activation. Restricted space and overpopulation are found in slum districts associated with urbanization, as seen in Asia particularly, in the workplace, in the school or home. Thus, the study of male mice exposed to social crowding appears to be a good model to investigate crowding-induced deterioration of diseases under relatively naturalistic social conditions.

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