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Lymphocyte-induced angiogenesis factor is produced by L3T4$^+$ murine T lymphocytes, and its production declines with age*

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Summary. Lymphocyte-induced angiogenesis factor (LIA) is a product of T lymphocytes which has been shown to stimulate new vessel formation. Because immune senescence most profoundly affects T lymphocyte functions, we suspected that LIA production would decline with age. An assay for angiogenesis stimulated by allogeneic reaction was performed by injecting spleen cells from young or old donor mice into the skin of irradiated allogeneic recipient mice. The spleen cells from young mice induced a significantly greater number of vessels than did cells from older mice. In additional experiments, spleen cells from young and old animals were treated with a monoclonal antibody GK1.5) directed at the L3T4 antigen on murine T helper lymphocytes. Such treatment significantly reduced the new vessel formation induced by young lymphocytes but had no effect on that induced by lymphocytes from old animals. Studies employing indirect immunofluorescence demonstrated that the proportion of L3T4$^+$ cells in the mononuclear fraction of splenocytes was nearly identical in both young and old mice. From these investigations we can conclude that (1) L3T4$^+$ lymphocytes are responsible for LIA production, and (2) production, like that of other T lymphokines, declines with age.

Introduction

Angiogenesis is a term which refers to the growth of new blood vessels from existing vasculature. It occurs commonly in normal animal tissue (embryonic growth, wound healing, local inflammatory reactions) [1, 9] as well as in abnormal tissue such as seen in the growth of neoplastic tissue [10]. We have recently observed that certain weakly antigenic murine tumors (B16 melanoma and Lewis Lung carcinoma) grow more slowly in old animals when compared to young [4–6]. When examined histologically, one characteristic of these tumors in old animals is their relatively low number of blood vessels [6]. It has been suggested that primary tumors and metastases are angiogenesis-dependent [7], and that every increase in cell population and in size is preceded by an increase in new capillaries.

Tumor-induced angiogenesis has been demonstrated in several experimental systems [8, 11, 12]. It is now evident that tumor cells are capable of releasing one or more soluble factors, tumor angiogenesis factors, which stimulate blood vessel growth and control the direction of propagation. The ability of these cells to release such factors has obvious advantages for the tumor tissue. Normal, competent lymphocytes, however, have also been shown to induce angiogenesis under certain circumstances such as when injected into an allogeneic host [2]. Lymphocyte-induced angiogenesis is mediated by a factor (lymphokine) produced by allo-reactive lymphocytes. The type of lymphocyte producing lymphocyte-induced angiogenesis factor (LIA) has not been absolutely characterized, however, we propose that LIA is a product of helper T lymphocytes and that its production declines with age.

Experimental evidence demonstrates that with age, T lymphocyte functions become diminished leading to decreased levels of lymphokine release [15, 19]. We speculate that LIA production contributes to tumor angiogenesis and that the decreased tumor vascularization in old animals results from an age-related decline in LIA production. Furthermore, we speculate that this decline is caused by a qualitative decrease in the level of lymphocyte function rather than by a decrease in the number of cells producing LIA. In an effort to explore these hypotheses the following experiments were performed.

Materials and methods

Animals. ICR outbred female mice and young (3 months) C57Bl/6 inbred female mice were obtained from Harlan Sprague Dawley, Madison, Wis. Old (24 months) C57Bl/6 females were generously provided for these experiments by Marc E. Weksler.

Lymphocyte procurement. For each experiment four donor mice were used. One ICR female mouse (4 months old) was used as a thymus donor (control) while two young C57Bl/6 females and one old C57Bl/6 female were used as spleen donors. The thymus and spleens were dispersed into single cell suspensions in RPMI-1640 medium (Gibco Laboratories, Grand Island, NY). The cells were washed twice and viable cells, determined by trypan blue exclusion, were adjusted to 1×10^6 cells/ml. From each suspension, 6×10^6 cells were isolated, half of which would subsequently be depleted of L3T4$^+$ (helper T) cells.

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Antibody. GK1.5 (American Type Culture Collection, Rockville, Md.) is a rat hybridoma which produces an IgG2b monoclonal antibody with specificity for the L3T4 antigen on murine helper T lymphocytes [3]. Culture supernatant from this hybridoma was generously provided for these studies by Robert Auerbach, University of Wisconsin-Madison.

L3T4$^+$ cell depletion. GK1.5 supernatant was added to the appropriate suspensions at a 1:10 dilution and allowed to incubated at 4° C for 30 min. Preliminary studies had indicated that these represented optimal conditions for specific cytotoxicity utilizing this antibody. The cells were then washed twice and rabbit complement (Cooper Biomedical, Malvern, Pa.) was added at a 1:10 dilution. After a 30 min incubation at 37° C, the cells were again washed twice. Reference suspensions were treated in the same manner substituting GK1.5 and complement with RPMI-1640.

Cell concentration adjustments. Reference groups were counted using trypan blue exclusion and viable concentrations were adjusted to 1×10^7 cells/ml. L3T4$^+$ depleted suspensions were adjusted to the same volume as their corresponding reference suspensions to assure consistency in the concentrations of other cell populations. All cell preparations were suspended in RPMI-1640 medium supplemented with 0.016% trypan blue to facilitate the location of the injection site.

Injections. For each experiment, 10 ICR female mice were exposed to 600 rad of total body irradiation (25 rad/min in air). Then 1 h later the mice were anesthetized with chloral hydrate at a dose of 5.25 mg/10 g of body weight. Each mouse then received four i. d. injections of four different cell suspensions. The injections were performed in a coded manner and blind. The code was set up in such a way that all suspensions were assayed at all four injections sites. The injection sites were all in the skin of the anterior abdominal wall. Each injection consisted of 0.1 ml of the appropriate cell suspension. Mice were then identified by ear marking.

Quantification. At 72 h after injection, mice were sacrificed and their skin separated from underlying tissue. After location of the injection sites, the interior surface of the skin was examined through a dissecting microscope at 25X magnification. All blood vessels which branched from the larger capillaries and which were directed toward the injection site were counted as described by Sidky et al. [17]. The results from various groups of cell suspensions were assessed for statistical significance by Student's *t*-test.

Immunofluorescence. Spleen cell suspensions were fractionated by centrifugation over Histopaque 1083 (Sigma Diagnostics, St. Louis, Mo.). Mononuclear cells were characterized for the presence of cell surface antigens by indirect immunofluorescence using anti-thy 1.2, anti-Lyt 2 (Becton-Dickinson, Mountain View, Calif.), and anti-L3T4 (American Type Culture Collction, Rockville, Md.). The second step reagent was fluorescein isothiocyanate-conjugated goat anti-rat IgG (Cooper Biomedical, Malvern, Pa.). Cytofluorography was performed using a FACS IV (Becton-Dickinson).

Results

LIA production: young vs, old

The assay system we used was designed to test for the angiogenic capacity of splenocytes from old and young donors compared to control cells. We also intended it to test for the importance of L3T4$^+$ lymphocytes in inducing angiogenesis. As shown in Table 1, splenocytes from young mice produced a significantly higher level of angiogenesis than did those from old (24.31 ± 0.70 vs 10.00 ± 0.88). Treatment with the antibody GK1.5 and complement did not significantly affect the angiogenic ability of splenocytes from old donors but significantly reduced the LIA ability of splenocytes from young mice (24.31 ± 0.70 vs, 10.46 ± 0.54) (Fig. 1). After GK1.5 treatment, all cell suspensions produced approximately the same LIA response.

T lymphocyte subsets in young and old mice

As shown in Table 2, our immunofluorescence study revealed that the proportions of various T lymphocyte sub-

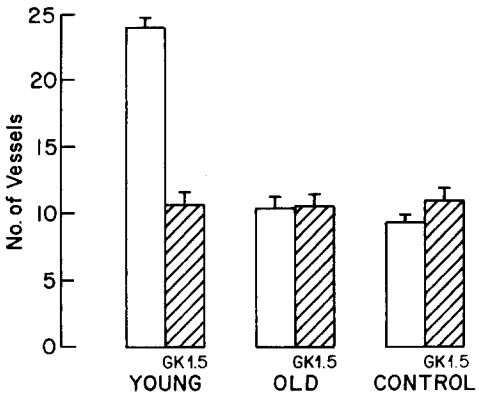

Fig. 1. Effects of GK1.5 in vitro treatment upon lymphocyte-induced angiogenesis. *Open bars* signify new vessel formation induced by untreated splenic cell populations from young or old allogeneic donors or from control (littermate) ICR thymocytes. *Hatched bars* reflect the effect of L3T4$^+$ cell depletion on the angiogenic response. Treatment of spleen cell populations from young animals (but not old) resulted in a reduced angiogenic response

Table 1. Allogeneic reaction-induced angiogenesis

Cell donor	Mean number of vessels	*n*
Young	24.31 ± 0.70 (29)	(29)
Old	10.00 ± 0.88 (13)	(13)
Control	9.21 ± 0.79 (14)	(14)

Table 2. Lymphocyte subsets in C57Bl/6 mice (expressed as percentages of cells in the mononuclear fraction of splenocytes)

Cell donor	Thy 1.2 (pan-T)	L3T4 (T_h)	Lyt 2 ($T_{c/s}$)
Young ($n = 3$)	51.8 ± 6.5	34.6 ± 3.6	37.5 ± 2.8
Old ($n = 3$)	44.5 ± 4.2	34.2 ± 0.9	33.9 ± 4.0

sets in the mononuclear fraction of splenocytes did not differ significantly between old and young mice.

Discussion

The role of the immune system in mediating angiogenesis is not completely understood. While studies have demonstrated the ability of lymphocytes to induce the formation of new blood vessels [17, 18] the roles of specific cell populations have not been reported. Furthermore, the effect of age on the ability of cells to induce angiogenesis has not been documented.

Most experimental tumors are at least weakly antigenic and, therefore, elicit an immune response. During the course of this response, helper T lymphocytes release lymphokines, one of which is believed to be LIA. While LIA has not been isolated, much experimental evidence supports its existence [2, 17, 18]. These experiments demonstrate that when immunocompetent mouse lymphocytes are injected i.d. into previously irradiated allogeneic recipient mice, local angiogenesis occurs. The i.d. injection of allogeneic lymphocytes produces a local graft-versus-host reaction which, within 2 to 3 days, results in visible blood vessel proliferation toward the injection site. Irradiation of the recipient prior to injection assures that any reaction which occurs is caused by antigenic stimulation of the injected cells.

Our hypothesis held that L3T4$^+$ lymphocytes (murine helper T lymphocytes) are responsible for the production of LIA and that production of this factor declines with age. The assay system we employed was, therefore, designed to test for two variables: (1) the importance of L3T4$^+$ murine lymphocytes in the induction of angiogenesis (LIA production) and (2) the effect of age on such induction.

The results, which are graphically represented in Fig. 1, indicate the importance of L3T4$^+$ lymphocytes in inducing angiogenesis. These data showed that while spleen cells from old mice were capable of inducing angiogenesis at a level only slightly above background (10.00 ± 0.88 vs 9.21 ± 0.79), spleen cells from young mice were able to induce such growth at a level approximately 2.6 times greater than the background level (24.31 ± 0.70 vs 9.21 ± 0.79). Splenocytes from young donors were, therefore, significantly more effective than splenocytes from old donors in mediating LIA. Furthermore, studies employing indirect immunofluorescence (Fig. 1) showed that the relative sizes of various T lymphocyte populations in the mononuclear fraction of splenocytes did not differ significantly between old and young mice. This indicates that the age-related decline in angiogenic capacity was due to a qualitative change in the level of lymphocyte function rather than to a decline in the number of lymphocytes.

Treatment of cells with the monoclonal antibody GK1.5 and complement demonstrated the importance of L3T4$^+$ lymphocytes in effecting LIA. While L3T4$^+$ depletion did not markedly affect the LIA ability of the control cells or of the splenocytes from old donors, it reduced the LIA effectiveness of splenocytes from young donors to a level only slightly above background (10.46 ± 0.54 vs 24.31 ± 0.70). While the old splenocytes and the control cells did not have significant LIA responses when untreated, the LIA responses of young spleen cells were lost with L3T4$^+$ depletion.

From these studies we can conclude that: (1) L3T4$^+$ T lymphocytes (murine helper T lymphocytes) are responsible for LIA production; and (2) the ability of L3T4$^+$ T lymphocytes to produce LIA declines with age. These conclusions may be one step in explaining the so-called age advantage of certain tumors. The finding that some weakly antigenic tumors grow more rapidly in young mice may be explained by an increased capacity for angiogenesis. Activated monocytes have been shown to produce angiogenesis factors [13, 14] and can recruit T lymphocytes capable of LIA production. Monocytes and T lymphocytes have also been demonstrated to infiltrate tumors [16], and the production of angiogenesis factors by these cells may result in enhanced tumor growth. For example, activation of L3T4$^+$ lymphocytes in young, tumor-bearing mice may lead to the production of LIA and the subsequent proliferation of new blood vessels into the tumor, facilitating its further growth. The age-reduced production of LIA, as we have demonstrated, may account for part of the decreased vascularization and slower growth of tumors in elderly hosts. The inhibition of LIA, therefore, may be useful for the inhibition of primary cancers or metastatic disease.

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