$\alpha_M\beta_2$ Integrin Modulator Exerts Antitumor Activity *In Vivo*

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Abstract. *Background: Leukocyte immunomodulation has great clinical potential as a therapy of inflammatory conditions and cancer. We have recently developed leukocyte $\alpha_M\beta_2$ integrin targeting small molecule (IMB-10) capable of inhibiting leukocyte migration and recruitment in vitro and in vivo. Materials and Methods: The purpose of this study was to investigate the potential anticancer effects of IMB-10 using U937 histiocytic lymphoma, OCI-AML-3 acute myeloid leukemia and HSC-3 tongue squamous cell carcinoma xenografts in athymic nude mice lacking T-lymphocytes. Results: IMB-10 therapy inhibited the growth of both leukemia and lymphoma xenografts and significantly prolonged the survival of the mice with lymphoma. Interestingly, IMB-10 also reduced host leukocyte infiltration in tumors and affected the invasion potential of squamous cell carcinomas. Conclusion: IMB-10 has potential as a therapy for leukocytic malignancies, particularly for lymphomas. Since it also inhibited HSC-3 carcinoma invasion, most likely by blockage of the tumor-infiltrating leukocytes, we suggest that the host inflammation process may affect tumor progression also in a T-cell independent manner.*

The integrins are group of at least 24 different heterodimeric transmembrane cell surface receptors associated with cell adhesion, migration, and both extra- and intercellular signaling (1). Circulating leukocytes are in a relatively inactive state but when chemoattractants or other signals from the immune system appear they become activated. Activated leukocytes use several cell surface proteins, including the integrins, to attach to the vascular epithelium when escaping from the blood stream. All leukocyte-specific integrins are composed of a β_2 chain (CD18) but they have different α-chains (CD11a, b, c, d) (2). In arrested or transmigrating leukocytes, the most important cell surface receptors are $\alpha_L\beta_2$ (CD11a/CD18, LFA-1) and $\alpha_M\beta_2$ (CD11b/CD18, Mac-1) integrins (3).

Studies with the leukocyte-specific $\alpha_L\beta_2$ and $\alpha_M\beta_2$ integrins have provided information on the integrin structure function relationship (4). There are two major mechanisms by which integrins bind to their ligands. About half of the integrins contain an inserted I-domain in the α-subunit as the major ligand-binding site whereas the rest of the integrins use both α and β chains to form a ligand-binding cavity. In activated leukocytes, several conformational changes enhance the affinity of integrin ligands. In addition, clustering of integrins on the leukocyte surface can enhance the affinity of the binding (3, 5, 6).

Integrin immunomodulation has great clinical potential when considering treatment of autoimmune diseases, inflammatory conditions and cancer. Leukocyte movement can be interfered either with inhibiting integrin ligand binding or with enhancing this interaction so that the ligand binds too strongly. Few inhibitors of β_2 integrins have been developed (7, 8). We have previously introduced leukocyte $\alpha_M\beta_2$ integrin targeting IMB-10 small molecule which attaches to the I-domain and stabilizes the integrin to an active conformation. Functioning like an agonist, IMB-10 significantly increases leukocyte adhesion potential and effectively inhibits their migration *in vitro*, as well as inflammation-mediated leukocyte recruitment *in vivo* (9). The inflammation process itself also plays an interesting role in tumor progression. The immune system and in particular the T-cell-mediated immune response have exerted an anti-tumorigenic effect in various types of cancer (10,11), but the effects may be dualistic especially in the early stages of tumor formation (12, 13). In this study the effects of IMB-10 on leukemia and lymphoma cell expansion *in vivo,* as well as on tumor infiltrating leukocytes in carcinoma xenografts were examined.

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Key Words: $\alpha_M\beta_2$ Integrin, carcinoma, leukemia, lymphoma, tumor-infiltrating leukocyte, invasion.

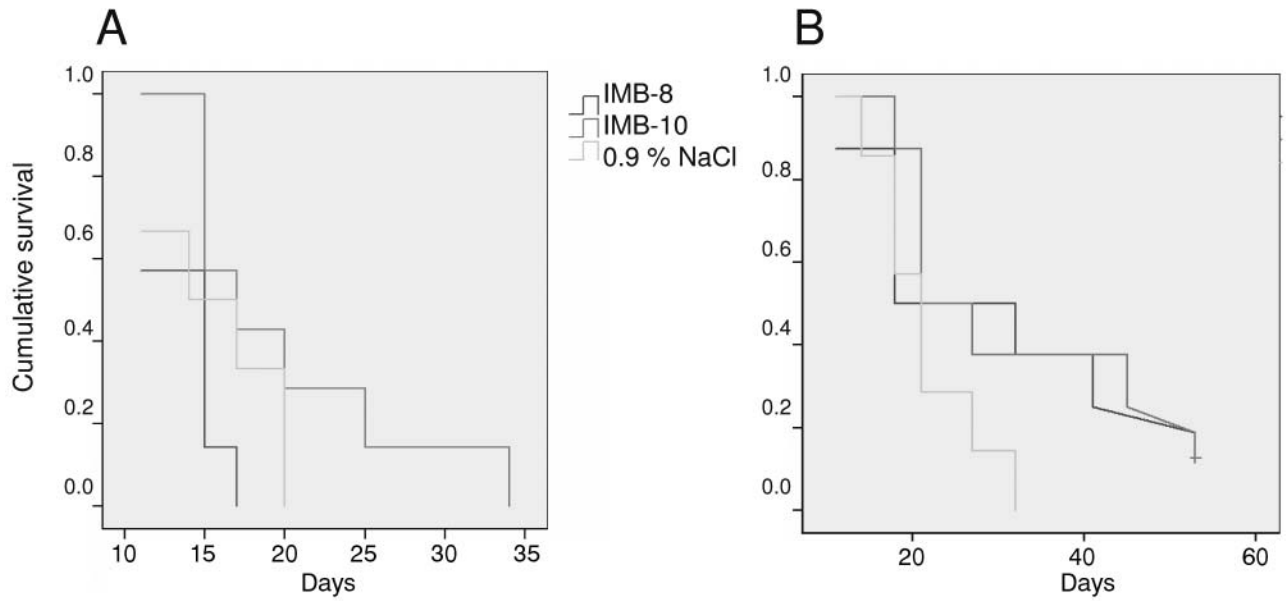

Figure 1. *Effects of IMB-10 on the growth of U937 lymphoma and OCI-AML-3 leukemia in athymic mice. (A) Kaplan-Meier survival of U937 tumor xenograft-bearing mice injected with IMB-8 or IMB-10 (n=7) in comparison to vehicle-treated mice (n=6). (B) Survival of OCI-AML-3 xenograft mice injected with IMB-8 or IMB-10 (n=8) compared to vehicle-treated mice (n=7). IMB-10-treated mice survived longer as compared to controls.*

Materials and Methods

Chemicals. Chemicals IMB-10 (3-(2-methylphenyl)-5-(3-phenyl-2-propen-1-ylidene)-2-thioxo-1,3-thiazolidine-4-one), CTRL-R (3-phenyl-5-(3-phenyl-2-propen-1-ylidene)-1,3-thiazolidine-2,4-dione), and IMB-8 (N-[3-allyl-4-(4-nitrophenyl)-1,3-thiazol-2(3H)-ylidene]-3-bromobenzenaminium bromide) were all purchased from ChemBridge, San Diego, CA, USA (9). CTRL-R and IMB-8 possess less than 10% and 25% of the activity of IMB-10, respectively.

Cells and cell cultures. The OCI-AML-3 acute myeloid leukemia, U937 histiocytic lymphoma (ATCC, Manassas, VA, USA), and HSC-3 tongue squamous cell carcinoma cells (JCRB Cell Bank, National Institute of Health Sciences, Japan) were cultured as described elsewhere (14-16).

Animal experiments. The animal experiments were approved by the Ethics Committee for the Animal Experiments at the University of Helsinki. The animals were maintained in the standard conditions for temperature and humidity, having food and water *ad libitum*. The U937 and OCI-AML-3 tumors were initiated by injecting $1x10^5$ cells in serum-free medium to both flanks of 6- to 8-week-old athymic nude female mice (weighing 20-24 g). The mice were intravenously (*i.v.*) injected with 100 μl chemicals at a 20 μg/ml concentration in physiological solution at days 4-8, 11-15, 18-22 and 25-28. HSC-3 tongue squamous cell carcinoma inoculation was performed with $2x10^6$ cells. HSC-3 tumor-bearing mice were treated with chemicals or saline five times a week from day four. Invasion and attachment of the tumor was evaluated by palpation and dissection of tumor area. The condition of the mice was inspected daily; the mice were weighed and tumor width and length were measured three times a week. Humane end-points were: weight loss over 15% or tumor diameter 10 mm. Euthanasia was performed by cervical dislocation.

Histochemistry. Histological analysis was performed by cutting 10-μm serial frozen sections of each tumor and as negative control 10-μm frozen sections of rat skin kindly provided by Dr. Taru Pilvi (Department of Pharmacology, University of Helsinki) were used. Thawed samples were fixed using ethyl alcohol and acetone, incubated with 1.6% H_2O_2 in methanol for 30 min to reduce endogenous peroxidase activity, washed three times with Tris-buffered saline (TBS), and then blocked with 10% rat normal serum and 2% bovine serum albumin (BSA) in TBS for 2h at room temperature. Specimens were incubated for 2 h in humidified dark chamber at room temperature using monoclonal rat anti mouse CD45 antibody-FITC-linked probes (Abcam, Cambridge, UK), which recognize all hematopoietic cell lines except mature erythrocytes, diluted 1:25 in 1% BSA in TBS. Before mounting, slides were counterstained for 10 s with Mayers hematoxylin followed by rinsing in running water for 10 min. From each tumor tissue slide, four of the most leukocyte-rich areas were photographed using an Olympus BX 61 microsope with magnification (x10), aperture stop, and exposure being kept constant. Leukocyte infiltration was analyzed by scanning the FITC-signal optical density of each visual area. The sections were also stained with hematoxylin and eosin (HE) for pathological analysis.

Gelatin zymography. Effect of IMB-10 on gelatinase production was analyzed by culturing HSC-3 cells in 6-well plates using serum-free culture medium supplemented with IMB-10 or its control CTLR-R up to 100 μM. After 96 h incubation, the culture medium was collected. Total proteins of the samples were determined using a Hitachi spectrophotometer and the Lowry method. The amount of

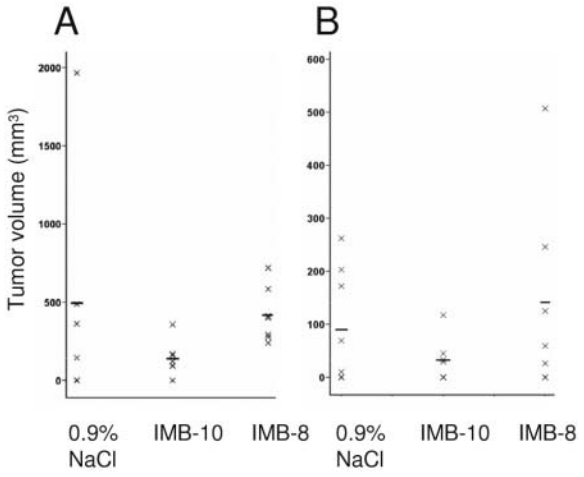

Figure 2. *Mean tumor volumes (mm³) from the last day when all mice were alive. IMB-10 treatment inhibited growth of both U937 (A) and OCI-AML-3 (B) xenografted tumors.*

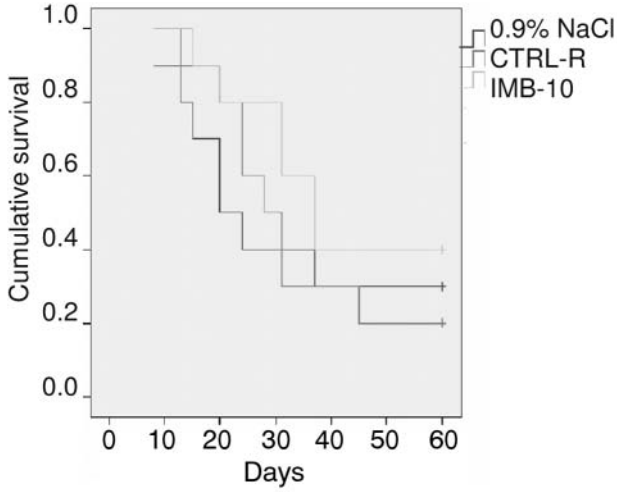

Figure 3. *Effect of IMB-10 on HSC-3 carcinoma xenografted athymic mice. Athymic nude mice (n=10 in each group) were inoculated with human tongue squamous cell carcinoma (HSC-3) cells. Kaplan-Meier survival of tumor xenograft-bearing mice injected five times a week with CTRL-R, IMB-10 or saline.*

gelatinolytic enzymes were analyzed using gelatin zymography and densitometric computer quantization as described elsewhere (14).

Analysis of the data. Results of the mouse survival were expressed as Kaplan-Meier curves and statistical significances were calculated using the Mantel-Cox test. Tumor volumes were calculated with the formula (Π/6) x A x B x B, where A is the length and B is the width of the tumor. Tumor volumes are from the last day when all animals were still alive and the results were statistically analyzed with the Mann-Whitney test. Tumor invasion data are expressed as numbers. The results were analyzed statistically (Pearson's Chi-square). Tumor infiltrating leukocytes are expressed as mean optical densities ± SD and analyzed statistically by variance analysis.

Results

IMB-10 treatment inhibits leukemia and lymphoma growth in vivo. We tested the effect of $\alpha_M\beta_2$ integrin stabilizing compound IMB-10 on tumor growth using U937 lymphoma and OCI-AML-3 leukemia xenografts in athymic mice. IMB-10 therapy given *i.v.* clearly prolonged the survival of mice bearing the tumors; however the results were statistically significant ($p=0.02$) only in the case of lymphomas (Figure 1). The tumors in IMB-10-treated mice were considerably smaller than in the controls (Figure 2), again differences in tumor growth were statistically significant only in the U937 lymphoma experiment ($p=0.006$). The less effective modulator of $\alpha_M\beta_2$ integrins, IMB-8, had no significant effect *in vivo*; in the leukemia model it slightly prolonged the survival as compared to the vehicle-treated group but the results were not statistically significant. Lymphoma and leukemia tumors in mice mainly showed a tendency for growing outwards and were poorly invasive, staying in the subcutaneous location even in later stages.

IMB-10 inhibits progression and invasion of HSC-3 tongue squamous cell carcinomas. Daily *i.v.* IMB-10 treatment reduced the growth of HSC-3 squamous cell carcinoma xenografts and prolonged the survival of mice (Figure 3). However, the differences in survival were not statistically significant ($p=0.2$). More interestingly, IMB-10 treatment effectively inhibited the invasion of HSC-3 cells to underlying muscle fascia (Figure 4). The macroscopic invasion of tumors in mice treated with IMB-10 was significantly less as compared to mice treated with 0.9% NaCl ($p=0.012$) or CTRL-R ($p=0.05$) (Table I).

IMB-10 blocks leukocyte infiltration of HSC-3 xenografts. Athymic nude mice lack a T-cell response but other inflammatory cells are present and functional. These cells can be detected by HE staining and more specifically using CD45 antibody staining (Figure 5A-D). From each tumor sample, four of the highest CD45 signal-containing visual areas were photographed and their optical density analyzed. In HSC-3 xenograft samples of the mice treated with IMB-10, the leukocyte infiltration was significantly reduced ($p=0.05$) as compared to the CTRL-R and 0.9% NaCl treated mice (Figure 5E).

No severe toxicity signs were detected during long term IMB-10 therapy. In lymphoma and leukemia experiments *i.v.*

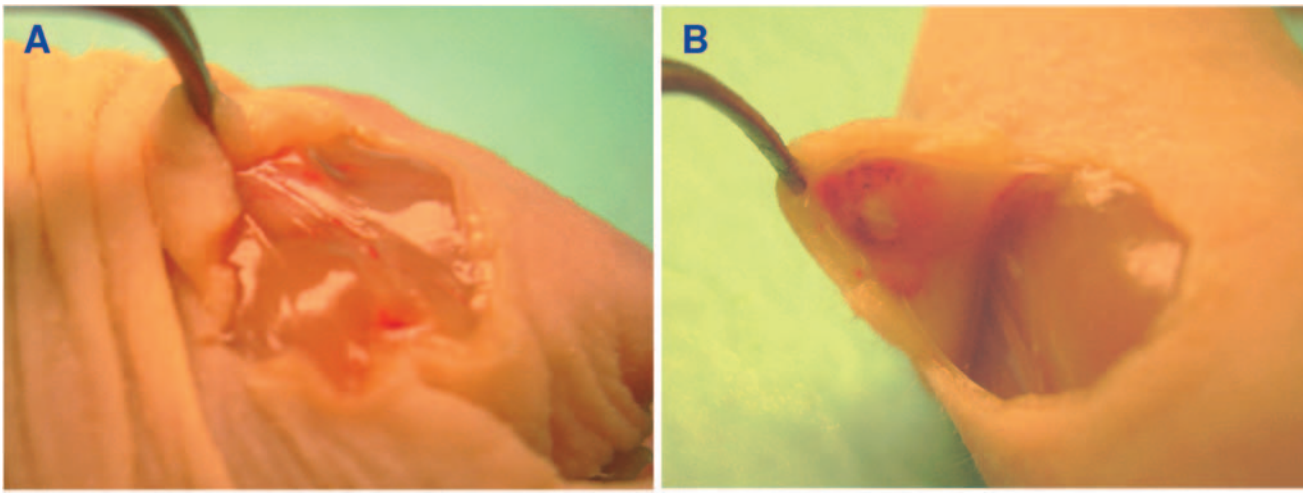

Figure 4. *HSC-3 tongue squamous cell carcinoma xenografts grow aggressively. Tumors of the mice treated with IMB-10 were less invasive to underlying muscle fascia and muscle (B) than these treated with control (A).*

administration of IMB-10 and controls continued almost for 30 days and no signs of toxicity were observed. In the carcinoma study, the treatment continued up to 60 days and no signs of toxicity such as weight loss, pain behavior, or organ anomalies were detected. In the CTRL-R-treated group, one mouse out of ten filled the criteria for euthanasia due to weight loss; in the dissection it was also found to have very large abdominal metastasis of the primary HSC-3 tumor, probably being the main cause of the symptom.

Table I. *Number of HSC-3 xenograft tumors invading the underlying muscle fascia of athymic mice.*

	Nature of HSC-3 tumor (n)	
Therapy	Invasive	Uninvasive
0.9% NaCl	13	7
CTLR-R	11	9
IMB-10	5	15

IMB-10 does not affect gelatinase function in carcinoma cells. Because there has been evidence that $\alpha_M\beta_2$ integrins and proMMP-9 (gelatinase B) are colocalized in activated neutrophils (17), we also tested whether IMB-10 has any direct effects on gelatinolytic protease in non-leukocytic cells. As judged by gelatin zymography, IMB-10 had no effects on the gelatinase production or activation by cultured HSC-3 cells (data not shown).

Discussion

We have previously shown that the $\alpha_M\beta_2$ integrin modulator IMB-10 effectively inhibits recruitment of leukocytes *in vivo* (9). Here we showed that IMB-10 is an effective therapeutic chemical for malignancies of leukocytic origin: it inhibited the growth of xenografted leukemias and lymphomas and prolonged the survival of the mice bearing these tumors. The effect of the chemical is probably based on its ability to prevent cell movement. The antitumor potential of IMB-10 on leukocytic malignancies was, nevertheless, less dramatic than expected on the basis of observations of our previous inflammation models, possibly due to the very rapid growth of these tumors. The less effective modulator of $\alpha_M\beta_2$ integrins, IMB-8, seemed to have no significant effect *in vivo* on the progression of leukocytic malignancies though in some *in vitro* experiments it had almost 25% of the activity of IMB-10 (9). As IMB-10 also reduced the growth and invasion of HSC-3 carcinoma xenografts and affected the leukocyte infiltration, we cannot exclude the possibility that the antileukemic effect of IMB-10 is also partly due to the blockage of host leukocytes.

Leukocyte integrin function can be interfered either with classical antagonism or stabilization of low affinity conformations of β_2 integrins (16, 18), or with agonism or stabilization of high affinity conformations as in the case of IMB-10. All leukocytes express a variety of β_2 integrins on their cell surface, but in particular neutrophils are enriched in $\alpha_M\beta_2$ integrins (6). Therefore, it is not surprising that IMB-10 potently inhibits neutrophil emigration in the model for acute inflammation (9). There is an ideal substrate binding-detachment ratio for effective cell movement and

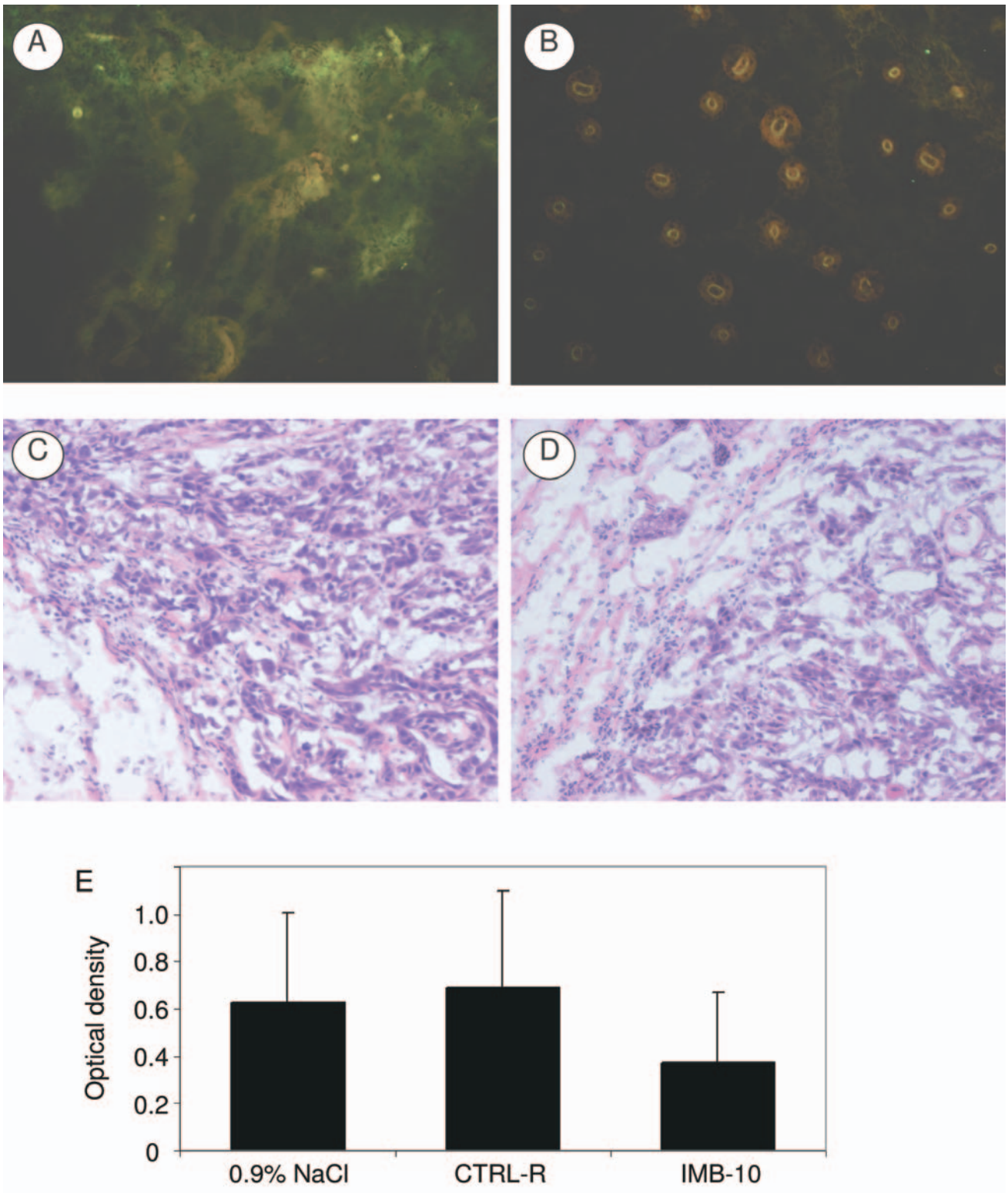

Figure 5. *IMB-10 blocks leukocyte infiltration into tumors. HSC-3 tumor samples were sectioned and stained with rat monoclonal antibody to mouse CD45 linked with FITC to evaluate leukocyte infiltration into tumors and with hematoxylin and eosin (HE) (A-D, magnification x10). Tumor sample of NaCl-treated mouse showed diffuse leukocyte infiltration (A), only few leukocytes infiltrated in the IMB-10-treated sample (B). Leukocyte infiltration can be seen also in HE-staining of the representative areas for control (C) and IMB-10 (D) showing predominant lymphocytic inflammation in the tumor stroma. Tumors of the mice treated with IMB-10 show about the half the leukocyte infiltration of the controls (E).*

even small changes can affect cell function (19). This is true also based on our studies with THP-1, U937 and OCI-AML-3 cell lines, since IMB-10 potently inhibited their movement though other β_2 integrin types are more predominant in them (2, 6, 9). However, there is evidence that interfering with $\alpha_M\beta_2$ integrin alone is not sufficient to inhibit neutrophil emigration (20).

Several studies confirm that cancer stromal interactions, as well as the host inflammatory system, plays a role in tumor progression: T- and natural killer (NK)-cells are commonly considered as having antitumor effects and macrophages tumorigenic effects (21, 22). Therefore anticancer strategies have been planned on immuno-modulation mainly focusing on vaccination with tumor antigens promoting the T-cell response (23). To our knowledge, no studies on leukocyte integrin targeting of solid tumors with small molecules have been performed, but it has been shown that inhibiting β_2 integrin function by specific antibodies can reduce leukocyte attachment to the tumor vasculature (24). In head and neck cancer, the inflammatory system has been shown to affect the aggressiveness of the disease, and most of the studies have focused on the T-cell-mediated host response (25). In this study we demonstrated that IMB-10 therapy also inhibited invasion potential of $\alpha_M\beta_2$ integrin-independent HSC-3 squamous cell tumors *in vivo*. The IMB-10-treated tumors also showed significantly diminished leukocyte infiltration in the tumor stroma indicating that the anti-invasive potential may be caused by the compound's ability to block integrin-mediated inflammatory cell recruitment. Although the athymic mice used in these experiments were lacking T-cells the other inflammatory cell populations in them were functional. All tumor samples showed chronic infiltration of macrophages and mononuclear leukocytes. These findings suggest that the inflammation process most likely plays an important role also in the carcinoma formation and progression.

In conclusion, the leukocyte $\alpha_M\beta_2$ integrin modulator IMB-10 is a promising prodrug for the development of therapies against leukocyte-originating malignancies. It can also reduce growth and invasion potential of squamous cell carcinomas, most likely by regulating the host inflammation response. No toxic effect of long-term IMB-10 therapy was observed, though the pharmacokinetics of IMB-10 still remain to be analyzed.

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