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Activating anti-CD40 antibodies induce tumour invasion by cytotoxic T-lymphocytes and inhibition of tumour growth in experimental liver cancer

Eduard Ryschich[a,c], *Angela Märten*[a,*,c], *Eduard Schmidt*[a,c], *Michael Linnebacher*[b], *Nicolas Wentzensen*[b], *Sven Eisold*[a], *Ernst Klar*[a], *Markus W. Büchler*[a], *Jan Schmidt*[a]

[a]*Department of General Surgery, University of Heidelberg, Im Neunenheimer Feld 110, 69120 Heidelberg, Germany*
[b]*Department of Molecular Pathology, University of Heidelberg, 69120 Heidelberg, Germany*

ARTICLE INFO

Article history:
Received 9 September 2005
Received in revised form 8 December 2005
Accepted 3 January 2006
Available online 27 March 2006

Keywords:
Liver cancer
Rat
Immunotherapy
CD40
Microcirculation

ABSTRACT

The aim of this study was to investigate the effects of an activating anti-CD40 antibody (aCD40Ab) on leukocyte adhesion to tumour vessels, leukocyte migration and tumour growth in experimental liver cancer. Morris-Hepatoma was induced by subcapsular inoculation of tumour cells in the liver of ACI-rats. On day 7 and 8 after tumour cell injection, one group of the animals received aCD40Ab. On day 13 the tumour volume was measured and intravital microscopy was performed quantifying leukocyte adherence in the liver. Furthermore, immunohistological analyses were performed. aCD40Ab-Treated animals showed increased leukocyte–endothelium interaction, demonstrated substantially more T- and natural killer (NK) cells in the tumour and had a distinctly decreased tumour volume. Our results show that treatment with aCD40Ab stimulates endothelial leukocyte adhesion in tumour vessels and migration of CD4 cells/CD8 T-cells and NK cells into the tumour and inhibits tumour growth. Thus, the CD40/CD154 pathway is a worthwhile target for adjuvant immunotherapy.

1. Introduction

The incidence of hepatocellular carcinoma (HCC) is increasing worldwide.[1,2] Hepatocellular carcinomas are seldom resectable and even if curative resection is possible the long term postoperative survival remains disappointing.[3] Current medical treatment concepts for HCC include chemotherapy, percutaneous injection of ethanol (PEI) chemotherapy with anthracyclins, medical treatment with anti-androgens or anti-estrogens and immunotherapy with interferon-alpha (INF-α).[2,9,10] All of these treatments, however, have not proven to be very effective[2,9,10] and there is an urgent need for other treatment modalities such as immunotherapy.

CD40 is a membrane protein of the tumour necrosis factor receptor family.[4] CD40 is expressed on antigen presenting cells (APC), including dendritic cells (DC), B cells, activated macrophages, and follicular dendritic cells.[4] APCs capture antigens, transport them to lymphoid organs, and present the antigens to CD4+ T-helper-cells and CD8+ cytotoxic-T-cells.[4] CD40–CD40 ligand (CD154) interactions play a pivotal role in activation of professional APC. CD154 enhances antigen presentation of CD40-expressing APC, followed by activation of effector T and natural killer (NK) cells. Activating CD40 antibodies mimic engagement of CD40 with CD154. This CD40/CD154 interaction plays a key role in the antigen-presentation.[4–6] CD40/CD154 interaction leads to an up-regulation of adhesion molecules

* *Corresponding author:* Tel.: +49 622 156 39890; fax: +49 6221 56 8240.
E-mail address: angela.maerten@med.uni-heidelberg.de (A. Märten).
[c] The first three authors contributed equally.
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doi:10.1016/j.ejca.2006.01.024

on endothelial cells and to stimulation of NK, CD4+ T-helper- and DCs.[4–6] DCs activate, via the CD40/CD154-interaction, CD4+ T-helper-cells.[5] Activated CD4+ T-helper cells produce diverse cytokines such as IL-12 and TNF-α, and stimulate CD8+ cytotoxic T cells directly.[5,6] These mechanisms can help the immune system to recognize and destroy the tumour as shown in previous reports.[5,6]

On the other hand the liver possesses specific immunological properties which could be used in anticancer therapy.[7] The intrahepatic concentration of lymphocytes is unusually high and represents 16–22% of the non-parenchymal cell pool in the liver.[8] Differential analysis of intrahepatic lymphocytes has shown a high number of cytotoxic CD8+T-cells.[8,9] The CD4:CD8 ratio in the liver is 1:3.5, whereas the ratio in the spleen is 1.8:1.[9]

The purpose of the presented study was to stimulate the potential of the large lymphocyte pool within the liver by improving antigen presentation of APC via CD40/CD154 interaction. By administration of activating CD40 antibodies (aCD40Ab), we mimicked the binding of CD154 ligand to its receptor CD40 to study the adhesion of leukocytes to tumour vessels, their migration and differential accumulation within the tumour. The effect of aCD40Ab on the growth of induced liver cancer was also studied using an established rat model of Morris Hepatoma.[10]

2. Materials and methods

2.1. Animals

Written consent for the experimental protocol and for all experiments was obtained from the responsible animal protection committee (Regierungspräsidium Karlsruhe, Karlsruhe, Germany). Fourteen male ACI rats (240–260 g) were included in the study. The rats were kept under standard conditions in a 12 h dark–light circle and given rat chow and water ad libitum; 18 h prior to the operative procedures, food was withheld, but they had free access to water. Two rats were excluded because they died under anaesthesia during tumour cells injection.

2.2. Tumour cell line

An established cell line of the hepatocellular carcinoma (hepatoma) in the rat, Morris Hepatoma (MH) 3924A (kindly provided by Prof. Dr. H. Wrba, Deutsches Krebsforschungszentrum, Heidelberg, Germany) was used. Morris Hepatoma 3924A was induced by the administration of *N*-2-fluorenyldiacetamide in ACI rats in 1951. It is a rapidly growing and poorly differentiated hepatocellular carcinoma.[11] The cell lines were grown in RPMI 1640 medium containing 100 μg/ml streptomycin and 10% heat-inactivated FCS. Cells were cultured for 4 days and were inoculated after separation into the liver (day 4 of the cell culture[10]).

2.3. Hepatoma induction and treatment

Two groups, each consisting of six rats, were studied. Anaesthesia was performed with intraperitoneal xylazinehydrochlorid (i.p.) (8 mg kg^{-1} body wt.) and intramuscular (i.m.) ketamine (40 mg kg^{-1} body wt.) The rats were placed in a supine position. An abdominal midline incision was performed. Animals received a subcapsular injection of 5 μl of MH 3924 cells solution (12×10^6 cells) into the left lobes of the liver. The abdominal midline incision was then closed by sutures. One group received 1 mg kg^{-1} body wt. stimulating monoclonal antibodies against CD40 subcutaneously (clone HM40-3, PharMingen, San Diego, CA) on the 7th and 8th day after tumour cell injection. The antibody is described to stimulate B cells and to induce expression of the B7 complex. Furthermore, dendritic cells are activated by this antibody.[14] No blocking antibodies are available at the present time. The control group received 1 mg kg^{-1} body wt. sc 0.9% NaCl on day 7 and 8 after injection of MH 3924 cells. On day 13, in vivo microscopy (IVM) was performed and the mean arterial pressure, as well as heart rate and arterial blood gases, were monitored. After the IVM the hepatoma was sampled for immunohistological analysis and the blood serum was collected for cytokine assessment.

2.4. In vivo microscopy (day 13)

Anaesthesia was performed with i.p. xylazinhydrochlorid (8 mg kg^{-1} body wt.) and i.m. ketamine (40 mg kg^{-1} body wt.) Erythrocytes were obtained from separate donor rats by heart puncture. They were labelled with FITC (Isomer I, Sigma No. F-1628, Sigma, Deisenhofen, Germany). Erythrocyte staining and tissue preparation were done according to the protocol from Sarelius and Duling.[11] Briefly, erythrocytes were washed in a glucose saline buffer and resuspended in a bicine-saline buffer (pH 6.8) containing FITC 9 mg × ml^{-1} and incubated at 25 °C for 3 h. The labelled erythrocytes were administered intravenously 30 min prior to the measurements. For labelling of leukocytes, 0.028 mg of rhodamine-6G was administered 5 min prior to the measurements as described by Baatz.[12]

A catheter was placed in the right jugular vein and the left carotid artery to supplement fluid loss and to monitor the mean systemic blood pressure, respectively. The rats were placed in a supine position and an abdominal midline incision was performed. In this way the surface of the liver and tumour was visible. The liver and the tumour were continuously superfused with 37 °C warm Ringer solution. The fluorescence microscope (Leitz, Wetzlar, Germany) was equipped with ×20 and ×40 water immersion lenses (Achroplan 20/0.75W and Achroplan 40/0.75W, Zeiss, Oberkochen, Germany). Epifluorescence was performed with an epifluorescence illuminator (Pleomak, Leitz) equipped with XBO 100W/2 short arcon Xenon lamp (Osram, Berlin, Germany), a 2-mm KG 1 heat-protecting filter, an excitation filter for FITC-labelled erythrocytes (450–490 nm) and an excitation filter for rhodamine-6G-labeled leukocytes (515–560 nm). Five post-capillary venules in the tumour were identified. The images of both excitation filters, which could be utilised in the same regions of interest (ROI), were recorded on videotape and off-line analysis then performed.

2.5. Off-line analysis of in vivo microscopy

The mean erythrocyte velocity, the mean vessel diameter and the leukocyte–endothelium interaction in tumour vessels

(20–40 μm) and in postcapillary venules of tumour-surrounding liver tissue were determined using a special software program (Cap Image®, Zeintl GmbH, Heidelberg, Germany). The erythrocyte velocity was evaluated by the frame-to-frame method.

Leukocytes that adhered to the vascular wall with high affinity (for more than 30 s) were determined as "stickers", and those that adhered with low affinity (for less than 30 s) were determined as "rollers". Leukocyte–endothelium interaction was expressed as the number of stickers and rollers/100 μm of vessel length.

Blood flow was calculated by the standard formula: $F(t) = \pi \times (D^2/4) \times \upsilon$ (with F, blood flow; D, mean venule diameter; υ, mean erythrocyte velocity).

Vascular wall shear rates were calculated by the standard formula: $Sr = 8 \times \upsilon/D$ (with D, mean venule diameter; υ, mean erythrocyte velocity).

2.6. Measurement of tumour volume

Tumour length (L) and width (W) were measured in histological sections and tumour volume was evaluated according to the formula:[14] Tumour volume (mm^3) = L (mm) × W^2 (mm)/2.

2.7. Immunohistochemistry

Tumour tissue was snap-frozen in liquid nitrogen. Thin sections were cut by cryostat (5 μm), fixed in acetone and hydrated in PBS. Endogenous peroxidase activity was blocked with H_2O_2 in methanol. The sections were incubated for 1 h with mAbs directed against rat CD4 (Dako, San Diego, CA), rat CD8 (Dako, San Diego, CA) and rat NK (anti-rat NKR-P1A, PharMingen, San Diego, CA). Isotype-matched rat IgG (PharMingen, San Diego, CA) was used as negative control. Frozen sections of the spleen were used as positive control. After washing in PBS, the sections were incubated for 10 min with a biotinylated secondary antibody (Dako, San Diego, CA) and coloured using an AP chromogen kit (LSAB, Dako, San Diego, CA).

Lymphocytes were counted in the tumour. The immunohistochemical images were digitally analysed. For this aim, four microscopic fields of 0.5 mm^2 were randomly chosen by light microscope (Leica DMRB, Leica GmbH, Germany), digitalized by a colour video camera (CF 20/4DX, Kappa GmbH, Gleichen, Germany) to histological images and saved in computer. Number of lymphocytes were counted and expressed per 1 mm^2 of surface.

2.8. Statistics

Results are expressed as the mean values ±SD from six rats per group. Analysis was performed using the Wilcoxon–Mann–Whitney-U-Test with $P < 0.05$ considered to be significant.

3. Results

3.1. Microcirculatory parameters

To study the effect of CD40 activation on liver lymphocytes we determined the rate of lymphocyte–endothelial interactions

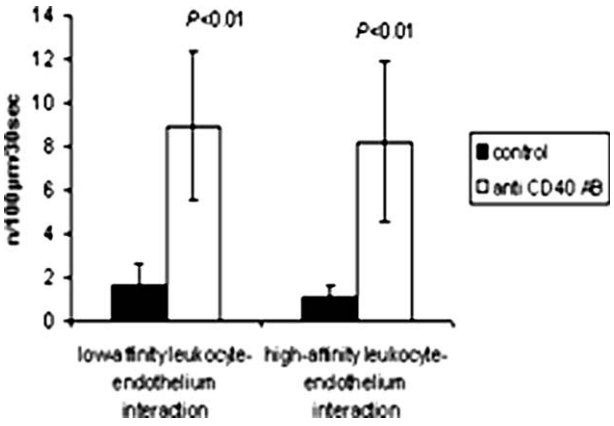

Fig. 1 – Adhesion of leukocytes. On day 13, intravital microscopy was performed quantifying leukocyte adhesion in postcapillary venules. Treatment of tumour-bearing rats with activating CD40 antibodies significantly increased the number of rolling and sticking leukocytes in the tumour vessels compared to controls (*: $P < 0.01$). Data presented as mean ± SD.

in the livers of treated and untreated mice. Both high- and low-affinity leukocyte–endothelium interaction in the tumour vessels were significantly increased (*$P < 0.01$) in the aCD40Ab-treated group compared with the control group (low-affinity leukocyte–endothelium interaction: 8.96 ± 3.40/100 μm/30 s in aCD40Ab vs. 1.63 ± 1.00/100 μm/30 s in controls; $P < 0.01$; high-affinity leukocyte–endothelium interaction: 8.21 ± 3.71/100 μm/30 s in aCD40Ab vs. 1.07 ± 0.55/100 μm/30 s in controls; $P < 0.01$) (Figs. 1, 2a and b).

Mean blood flow and erythrocyte velocity were determined as control. The mean erythrocyte velocity and calculated mean blood flow in the tumour vessels were not different compared with the control group (vessel diameter 32.2 ± 4.2 μm in controls, 31.8 ± 2.8 μm in aCD40Ab) (erythrocyte velocity: 1.69 ± 0.24 mm/s in controls, 1.47 ± 0.31 mm/s in aCD40Ab) (blood flow: 1.47 ± 0.51 nl/min in controls, 1.22 ± 0.29 nl/min in aCD40Ab; data not shown).

3.2. Stimulation of CD4+, CD8+ and NK cells

To determine the effector cells responsible for possible tumour growth suppression, we performed immunohistological analysis by quantitation of CD4+, CD8+ and NK cells in the tumour.

The number of both the CD4 and CD8 positive cells were significantly increased (*$P < 0.01$) in the aCD40Ab-treated animals in comparison with the untreated animals (CD8: 386 ± 141 cells/mm^2 in controls vs. 987 ± 454 cells/mm^2 in aCD40Ab, CD4: 324 ± 224 cells/mm^2 in controls vs. 1365 ± 472 cells/mm^2 in aCD40Ab; Figs. 3, 4a–d).

There was a high number of NK cells in the tumour in both treated and untreated animals (Figs. 5a and b). After the treatment with stimulating CD40-antibodies the number of intratumoural NK cells increased significantly ($P < 0.01$) in comparison to the untreated animals (260 ± 91 cells/mm^2 without therapy vs. 534 ± 147 cells/mm^2 with therapy; Fig. 3).

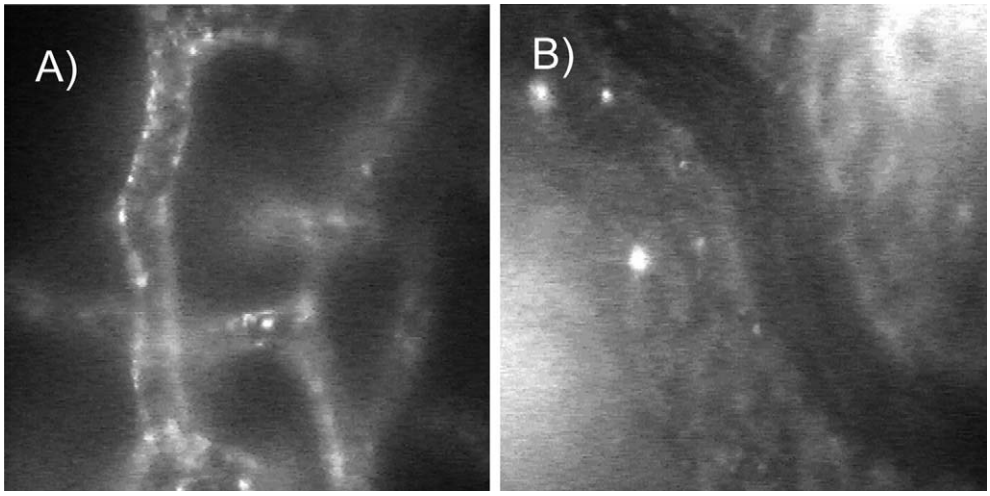

Fig. 2 – (A,B) Intravital microscopy of high-affinity leukocyte adhesion. Treatment of tumour-bearing rats with activating anti-CD40 antibodies resulted a significant increase of high-affinity leukocyte adhesion in blood vessels within the tumour. (A) Treated tumour, (B) non-treated tumour ($P < 0.01$).

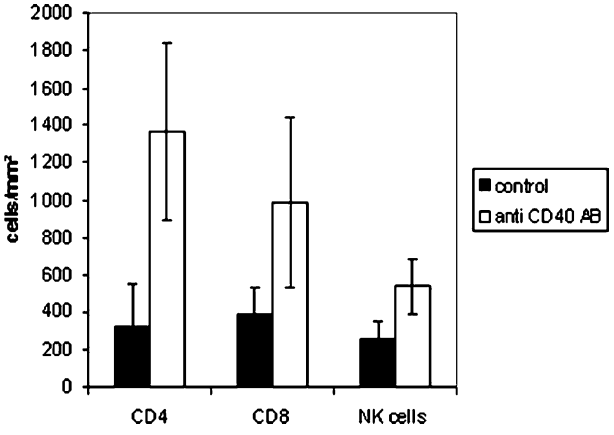

Fig. 3 – Quantification of intratumoural lymphocytes. On day 13 fresh tissue was snap-frozen and immunohistochemistry was performed quantifying CD4+ and CD8+ leukocytes within the tumour. Treatment of tumour-bearing rats with activating anti-CD40 antibodies significantly increased the number of CD4+ and CD8+ leukocytes within the tumour compared to controls ($P < 0.01$). Data presented as mean ± SD.

3.3. *Tumour growth in vivo*

The tumour length, width and calculated tumour volume were significantly decreased in the aCD40Ab-treated animals compared with the control animals. The tumour length in the treated animals was 9.5 ± 1.8 mm vs. 12.6 ± 3.2 mm in the untreated animals, the width was 4.9 ± 0.9 mm vs. 6.3 ± 0.9 mm and the tumour volume was 102 ± 42.5 mm^3 vs. 256.6 ± 107 mm^3 ($P < 0.01$; Fig. 6).

The presence of metastases were examined in all animals. Two of the untreated animals had local metastases (beyond 10 mm away from the primary tumour). In contrast, none of the treated animals demonstrated metastases (data not shown).

4. Discussion

It was previously demonstrated that monoclonal antibodies against CD40 activate APCs, enhance tumour antigen presentation and stimulate CTLs.[13,14] We found that stimulating monoclonal antibodies against CD40 are an effective treatment against liver cancer in our rat model by inhibiting tumour growth. Our data suggests that the observed effects are due to a stimulation of leukocyte adhesion to tumour vessels, an activation of CD8+, CD4+ and NK cells and their migration into the tumour. By simple subcutaneous administration of activating anti-CD40 antibodies we found that the tumour volume in the treated animals was 60% smaller than in the untreated animals. Two of the untreated animals had local metastases while none of the treated animals had any metastases. These novel findings demonstrate that immunotherapy per se can be effective in the treatment of experimental liver cancer. Although the final proof could only be done by administration of blocking anti-CD40 antibodies, this step could not be performed since blocking antibodies are not available at the present time.

Leukocytes are transported to the liver by the blood stream and come into contact with endothelial cells.[15] At first, the collision with endothelial cells leads to leukocyte rolling only if they express selectins and their ligands.[15] In the next level of activation, leukocytes respond to selectins by initiating signalling cascades which lead to up-regulation of integrins such as ICAM-I and VCAM-I[15] and consecutive firm endothelium–interaction, i.e. sticking.[15] After this step they migrate into the interstitium and can then participate in the local immune response.[15] CD40–CD154 interactions up-regulate intercellular adhesion molecules such as E-selectin, VCAM-I and ICAM-I on endothelial cells and enhance leukocyte–endothelium interaction.[16]

In vivo fluorescent microscopy is suited to obtain quantitative and qualitative information about local microcirculation and leukocyte adherence was shown in previous studies.[17] In the present report we observed a high number of leukocytes

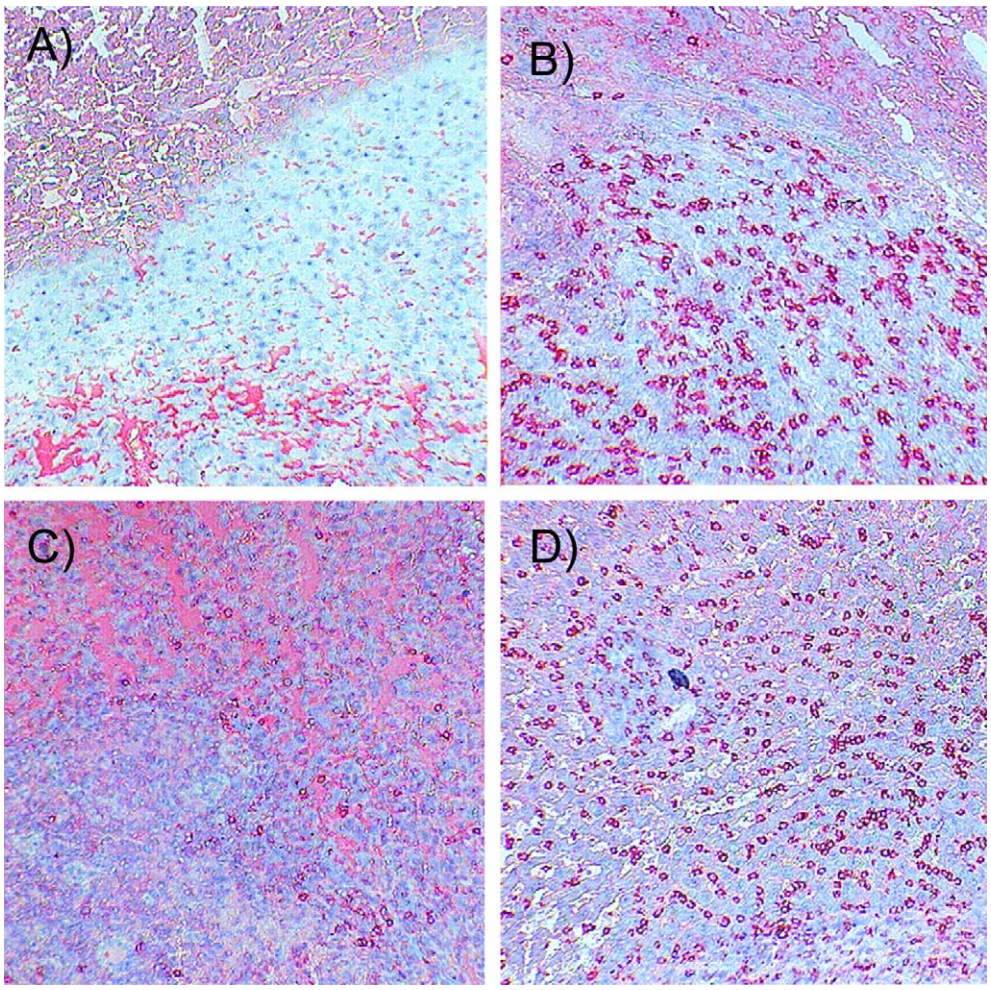

Fig. 4 – (A–D) Immunohistochemistry of intratumoural lymphocytes. Treatment of tumour-bearing rats with, activating anti-CD40 antibodies resulted in a significant increase of the number of CD4+ (A,B) and CD8+ (C,D) leukocytes within the tumour ($P < 0.01$). (A,C) Control tumours, (B,D) treated tumours. Immunohistochemistry of CD4+ and CD8+ leukocytes. Magnification ×250.

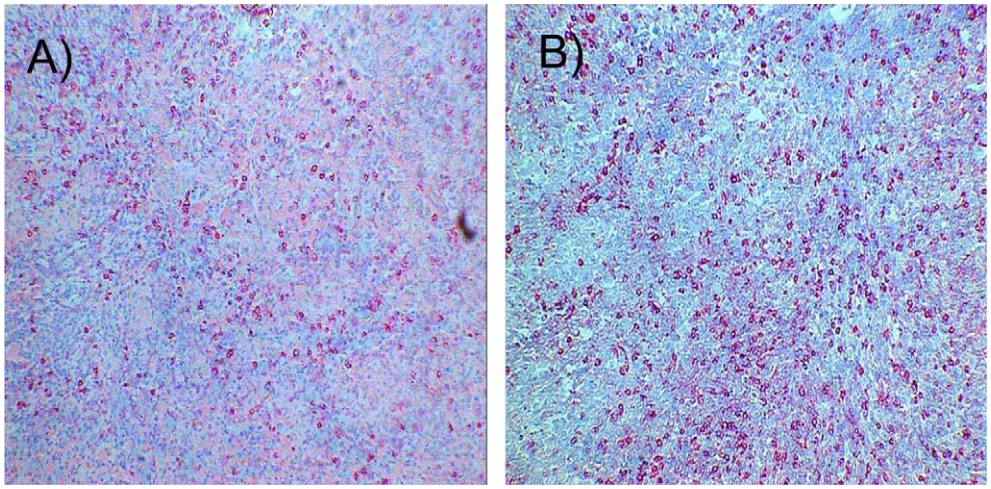

Fig. 5 – (A,B) Immunohistochemistry of intratumoural NK cells. Treatment of tumour-bearing rats with activating anti-CD40 antibodies resulted in a significant increase of the number of NK cells within the tumour. (A) Control tumour, (B) Treated tumour ($P < 0.01$). Immunohistochemistry of NK cells. Magnification ×250.

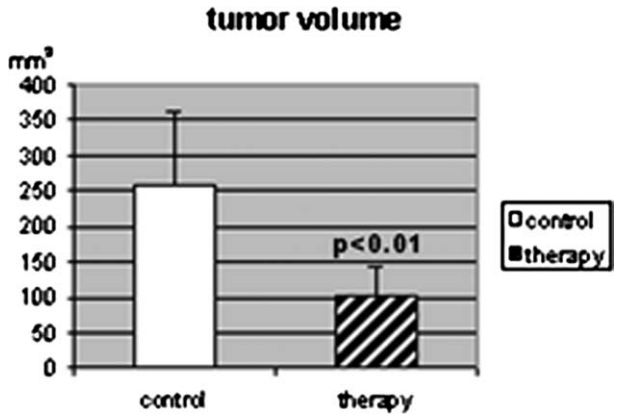

Fig. 6 – Tumour size in treated and control rats. On day 13 the tumour length (*L*) and width (*W*) were measured and tumour volume was evaluated according to the formula: tumour volume (mm^3) = *L* (mm) × W^2 (mm)/2. Treatment of tumour-bearing rats with activating CD40 antibodies significantly decreased the tumour volume compared to controls (*: $P < 0.01$). Data presented as mean ± SD.

adhering to the vascular wall of tumour vessels with low affinity (rollers) 5 days after the treatment with aCD40 antibodies (day 13 after tumour inoculation). The number of leukocytes adhering to the vascular wall of tumour vessels with high affinity (stickers) was also significantly increased. Minor amounts of sticking leukocytes in the control group represent a baseline status and confirm data presented in other studies.[18] Since the blood flow and calculated wall shear rates were similar in the tumours of treated and untreated animals the higher number of rollers and stickers in the aCD40Ab-treated group is a sign of true activation.[15] The observation that leukocyte adhesion in malignant tumour vessels (without stimulation) is reduced compared to healthy vessels was previously documented.[19]

Cytotoxic T-cells (CD8+) are responsible for rejection of malignant cells and are activated by CD4+ T-cells.[13,20] Previous studies demonstrated that both CD4+ and CD8+ T cells are important for an effective antitumour immune response.[13] Interactions between CD40 ligand and CD40 on CD4+ T cells and APC are essential for the priming of CD8+ cytotoxic T-cells to lyse tumour cells.[4] Furthermore, activating anti-CD40 antibodies stimulate APC directly.[4] Activated APC prime CD4+ and CD8+ T-cells to destroy the tumour[4] and as we used an immunogenic tumour model we found moderate amounts of CD 4+ and CD8+ T-cells in the tumour of untreated animals. This confirms previous studies showing a high number of CD8+ cytotoxic T-cells and CD4+ T-cells in the liver.[9] However, CD4+ and CD8+ cells increased significantly after treatment with activating anti-CD40 antibodies. These differences correlate with the observations of the high number of rollers and stickers in the tumour venules. After aCD40Ab therapy, a high number of CD8+ T-cells infiltrated the tumour however there were even more CD4+ T-cells that had migrated into the tumour. These observations underline the importance of CD4+ T-cells in antitumour immune response and correspond to data that have shown direct activation of CD4+ T-cells via CD40–CD40L interaction.[21]

Another mechanism that could be involved in the inhibition of tumour growth after activating anti-CD40 antibody treatment is the activation of NK cells.[22] The murine liver contains a large number of NK cells which are potent effector cells against tumours. Activation of NK cells can consequently lead to tumour lysis which results in the release of apoptotic and necrotic bodies.[22,23] The necrotic bodies are then taken up and presented to T cells.[23] NK cells express only rarely the CD40 ligand and no CD40. However, it was shown that treatment with activating anti-CD40-antibodies resulted in NK cell activation.[22] It was suggested that aCD40Ab stimulates NK cells indirectly, possibly by cytokine production upon CD40 coupling to APCs.[22] Tumours often do not express MHC-I-proteins and can escape the CD8+ T-cells immune response.[24] NK cells in comparison to cytotoxic CD8+ T-cells do not require MHC class I molecules to recognise the tumour, but can lyse tumours that do not express these proteins.[22] In our study we saw a significant increase of infiltrating NK cells after therapy with aCD40Ab. Thus, it seems conceivable that NK cells were activated indirectly via aCD40Ab action, migrated into the tumour and contributed to the inhibition of tumour growth.

One important mechanism involved in the regulation of immune responses after CD40 stimulation is an increased cytokine secretion such as TNF-α.[25] TNF-α is typically increased after CD8+- and NK cell activation. TNF-α increases immunogenicity of tumour cells, stimulates cytokine secretion by effector lymphocytes, activates leukocytes and inhibits tumour growth.[26]

In conclusion, we have shown that activating CD40 antibodies induce a significant antitumour effect in rat Morris Hepatoma and that this is probably mediated by the immune effector function of CD4+, CD8+ and NK cells.

Conflict of interest statement

The authors state that there are no conflicting financial interests.

Acknowledgement

This study has been supported by the Deutsche Forschungsgemeinschaft SFB 405.

REFERENCES

1. Lin DY, Lin SM, Liaw YF. Non-surgical treatment of hepatocellular carcinoma. *J Gastroenterol Hepatol* 1997;**12**:S319.
2. Ohmoto K, Tsuduki M, Shibata N, et al. Percutaneous microwave coagulation therapy for hepatocellular carcinoma located on the surface of the liver. *Am J Roentgenol* 1999;**173**:1231.
3. The Liver Cancer Study Group of Japan. Primary liver cancer in Japan: clinicopathologic features and results of surgical treatment. *Ann Surg* 1990;**211**:277–87.
4. Grewal IS, Flavell RA. CD40 and CD154 in cell-mediated immunity. *Annu Rev Immunol* 1998;**16**:111–35.
5. Costello RT, Gastaut J-A, Olive D. What is the real role of CD40 in cancer immunotherapy? *Immunol Today* 1999;**20**:488.

6. Nakajama A, Kodama T, Morimoto S, et al. Antitumour effect of CD40 ligand: elicitation of local and systemic antitumour responses by IL-12 and B7. *J Immunol* 1998;**161**:1901.
7. Mehal WZ, Azzaroli F, Crispe IN. Immunology of the healthy liver: old questions and new insights. *Gastroenterology* 2001;**120**:250–60.
8. Fleming KA. The anatomy of the normal liver and the hepatic lymphocyte. In: Crispe IN, editor. *T lymphocytes in the liver; immunobiology, pathology and host defense*. New York: Wiley-Liss; 1999. p. 1–15.
9. Norris S, Collins C, Doherty DG, et al. Resident human hepatic lymphocytes are phenotypically different from circulating lymphocytes. *J Hepatol* 1998;**28**:84–90.
10. Maksan SM, Paulo H, Ryschich E, et al. In vivo assessment of angioarchitecture and microcirculation in experimental liver cancer: a new model in rats. *Dig Dis Sci* 2003;**48**(2):279–90.
11. Morris HP, Slaughter LJ. Historical development of transplantable hepatomas. In: Morris HP, Criss WE, editors. *Adv Exp Med Biol*, vol. 92. New York, London: Plenum Press; 1978. p. 1–19.
12. Baatz H, Steinbauer M, Harris AG, et al. Kinetics of white blood cell staining by intravascular administration of rhodamine 6G. *Int J Microcirc Clin Exp* 1995;**15**:85–91.
13. Schoenberger SP, Toes RE, van der Voort EI, et al. T-cell help for cytotoxic T lymphocytes by CD40–CD40L interactions. *Nature* 1998;**393**:480–3.
14. Ridge JP, Di Rosa F, Matzinger PA. Aconditioned dendritic cell can be temporal bridge between a CD4+ T-helper and T-killer cell. *Nature* 1998;**393**:474–8.
15. Panes J, Granger DN. Leukocyte–endothelial cell interactions: molecular mechanisms and implications in gastrointestinal disease. *Gastroenterology* 1998;**114**:1066–99.
16. Thienel U, Loike J, Yellin MJ. CD154 (CD40L) induces human endothelial cell chemokine production and migration of leukocyte subsets. *Cell Immunol* 1999;**198**:87–95.
17. Goldsmith HL, Spain S. Margination of leukocytes in blood flow through small tubes. *Microvasc Res* 1984;**27**:204–22.
18. Schmidt J, Ryschich E, Volker D, et al. Vascular structure and microcirculation of experimental pancreatic carcinoma in rats. *Eur J Surg* 2000;**166**:1102–4151.
19. Griffioen AW, Damen CA, Blijham GH, et al. Tumour angiogenesis is accompanied by a decreased inflammatory response of tumour-associated endothelium. *Blood* 1996;**88**:667–73.
20. French RR, Chan HTC, Tutt AL, et al. CD40 antibody evokes a cytotoxic T-cell response that eradicates lymphoma and bypasses T-cell help. *Nat Med* 1999;**5**: 548–53.
21. Baxevanis CN, Voutsas IF, Tsitsilonis OE, et al. Tumour-specific CD4+ T lymphocytes from cancer patients are required for optimal induction of cytotoxic T cells against the autologous tumour. *J Immunol* 2000;**164**: 3902–3912.
22. Turner JG, Rakhmilevich AL, Burdelya L, et al. Anti-CD40 antibody induces antitumour and antimetastatic effects: the role of NK cells. *J Immunol* 2001;**166**:89–94.
23. Fernandez NC, Lozier A, Flament C, et al. Dendritic cells directly trigger NK cell functions: cross-talk relevant in innate anti-tumour immune responses in vivo. *Nat Med* 1999;**5**:405–11.
24. Ockert D, Schmitz M, Hampl M, et al. Advances in cancer immunotherapy. *Immunol Today* 1999;**20**:63–5.
25. Gruss HJ, Hirschstein D, Wright B, et al. Expression and function of CD40 on Hodgkin and Reed–Sternberg cells and the possible relevance for Hodgkin's disease. *Blood* 1994;**84**:2305–14.
26. Aggarwal BB, Natarajan K. Tumour necrosis factors: developments during the last decade. *Eur Cytocine Netw* 1996;**7**:93–124.