Peptides Against Mac-1 Do Not Sufficiently Target Leukemia or Lymphoma *In Vivo*

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Abstract. *Background: β2 Integrins (cluster of differentiation-18, CD18) are expressed only by leukocytes and serve as cell surface receptors, being involved both in inside-out and outside-in signalling, and in cell movement. Therefore, they are interesting targets for therapeutic intervention. Phage display-derived inhibitory peptides against $\alpha_M\beta_2$ integrins (macrophage-1 antigen, Mac-1) have been found to be effective in preventing leukocyte movement in vitro and in vivo but little is known regarding their ability to target leukaemia and lymphoma in vivo. Materials and Methods: Athymic nude mice were inoculated with human THP-1 acute monocytic leukemia (AML-M5 variant), U937 diffuse histiocytic lymphoma, and OCI-AML-3 acute-myeloidic leukemia cells, and then treated with Mac-1-inhibiting peptides ADGACILWMDDGWCGAAG (DDGW) or CPCLLGCC fused with green fluorescent protein (LLG-GFP). Results: Mac-1-inhibiting DDGW peptide had no effect on leukemia and lymphoma burden in mice, and LLG-GFP fusion did not home to leukemia cells in vivo. Conclusion: Although peptides against Mac-1 are promising drugs and diagnostic tools based on earlier experiments in inflammation they exhibit compromised biological avidity as a therapeutic and diagnostic means for leukaemia and lymphoma.*

In general four cell-surface protein families are responsible for motility-associated cell–cell and cell–matrix interactions: cadherins, selectins, immunoglobulin superfamily adhesion proteins, and integrins (1). Integrins are cell surface-bound glycoproteins composed of transmembrane subunits α and β, and are essential in various steps of cell adhesion, movement and signalling (2). β2 Integrins (cluster of differentiation-18, CD18) are expressed only in cells of leukocytic origin (3). The α-subunits capable of forming dimers with β_2 are α_L (also known as cluster of differentiation-11a or CD11a and lymphocyte function-associated antigen-1 or LFA1), α_M (also known as cluster of differentiation-11b (CD11b); macrophage-1 antigen (Mac-1), or complement receptor-3 (CR3)), αX (also known as cluster of differentiation-11c (CD11c); complement receptor-4 (CR4)) and αD (cluster of differentiation-11d (CD11d)) (3, 4). All β_2 integrins recognise several cell surface, extracellular matrix (ECM), and plasma proteins as well as inter-cellular adhesion molecules (ICAM). They are stored in the intracellular space, from where they are rapidly recruited into the cell membrane upon cell activation (5). β_2 Integrins also serve as complement receptors (CR3, CR4) recognising complement fragments, such as C3b and iC3b, and contribute especially in the function of phagocytic cells. Extravasation and migration of leukocytes is mediated by several distinct molecules such as selectins, but contact between endothelial cell ICAM-1 and β_2 Integrins are involved especially in the arrest of the transmigrating leukocyte (6, 7). Since β_2 Integrins are expressed only in myeloid and lymphoid cells they are also interesting targets for the clinical intervention in inflammatory conditions, leukaemias, and lymphomas. Only a few non- Arginyl-Glycyl-Aspartat –repeat (RGD) recognition-based Integrin inhibitor strategies have so far been developed. β_2 Integrin-targeting inhibitors and immunomodulators have been of special interest and the results of their use have been promising (8-14). Phage display techniques have also been successfully used to

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characterize synthetic inhibitory peptides against $\alpha_M\beta_2$ Integrins; discovered peptides CPCLLGCC (LLG) and ADGACILWMDDGWCGAAG (DDGW) are effective in preventing leukocyte attachment *in vitro* and inflammation *in vivo* (9-11). We have also developed non-peptide-based immunomodulators of leukocyte Integrins which are also capable of inhibiting lymphoma growth *in vivo* (12, 13). Some recent *in vivo* results of DDGW-based anti-leukaemic modulators have also been promising (15) but further bioavailability data are still needed. Since β_2 Integrins are expressed only in haematopoietic cells, they are also an interesting target when developing diagnostic strategies. For example, patients suffering from infectious diseases of unknown origin or certain haematological malignancies such as intracranially-manifested leukaemia, could benefit from mapping and localization of tissues and cells bearing activated β_2 Integrins.

Materials and Methods

Cells and cell cultures. The human THP-1 monocytic leukaemia (AML-M5 variant), U937 diffuse histiocytic lymphoma, and OCI-AML-3 acute-myeloidic leukaemia cells were cultured in an Roswell Park Memorial Institute culture medium (RPMI) supplemented with 25 μM of *4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid* (HEPES), 100 units/ml penicillin, 1% L-glutamin, 50 μM β-mercaptoethanol with or without 10 % fetal bovine serum (FBS) all from Life Technologies, Carlstad, CA, USA. The expression of β_2 Integrins was analyzed from cells' prior use by fluorescence-activated cell sorting (FACS) as described in (16).

Peptide. Bioactive peptide ADGACILWMDDGWCGAAG (DDGW) was a generous gift from Dr. Erkki Koivunen (University of Helsinki, Finland). Lyophilized peptide was pre-diluted in 10 mg/ml in 10% dimethyl sulfoxide (DMSO)/90% saline, which was then further diluted to 2 mg/ml in saline prior to use.

CPCLLGCC-peptide – Green Fluorescent Protein (LLG-GFP) fusion. Fusion between CPCLLGCC (LLG) and green fluorescent protein (GFP) was prepared and purified as described in (17). The controls were prepared similarly as described in (18, 19).

Animal experiments. The animal experiments were performed as approved by the Ethics Committee for animal experiments at the University of Helsinki, and by the Ethics Committee for Animal Experimentation in the State Provincial Office of Southern Finland. The THP-1, U937 and OCI-AML-3 leukaemia cells were xenografted as described elsewhere (13). Briefly, localized leukaemia cells were inoculated by injecting 5×10^5 cells in 200 μl of serum-free medium into both flanks of 6 to 8–week-old athymic nude nu–female (Harlan, the Netherlands). In the DDGW study, the mice (n=8) were treated on days 4-8 either with 200 μg DDGW (2 mg/ml in 100 μl) or with control solution. In the monitoring period, criteria for euthanasia were significant weight loss or tumor diameter >10 mm. Tumour volumes were calculated with the formula (Π/6)XAXBXB, where A is the length and the B is the width of the tumor. Tumor invasion into underlying muscle fascia and clinical metastasis was evaluated as described elsewhere (13). In the LLG-GFP-fusion tumour-targeting study, the THP-1 tumours were initiated as described previously (19). The tumours were allowed to grow 18 days. The mice were injected either with saline, LLG-GFP, or non functional Ala-LLG-GFP or His-GFP (all at 1 mg/ml in 100 μl of PBS). The fusion peptides were allowed to redistribute one hour after injection and then the mice were euthanized for tumor fluorescence studies.

Tumor fluorescence analysis. The tumors were embedded in Tissuetek-OCT (Sakura, Japan) and frozen. Frozen sections (10 μm) were cut with a microtome. Tumor tissue was confirmed by histological analysis as described elsewhere (19, 20) and sample fluorescence detected with a Axiovert 200M microscope (Zeiss, Germany) using filters for GFP ex. 395±40 nm/em. 510±40 nm (Chroma Technology Corp., Bellows Falls, VT, USA). Fluorescence of each sample was quantified from the three most intensive areas (magnification, ×400) with Quantity-One program (Bio-Rad, CA, USA).

Buffy coat-targeting in vitro. Buffy coat was obtained from the Finnish Red Cross; it was isolated from the blood of donators on the day of experiment's. Buffy coat was diluted 1:100 with PBS, activated with 100 nM Phorbol 12,13-dibutyrate (PDBu) for 15 min at room temperature, and incubated with LLG-GFP, Ala-GFP or His-GFP constructs for 30 min. After incubation, the samples were washed four times with PBS and cell smears were prepared on microscope slides using coverslips; fluorescence was observed using an Olympus Axiovert microscope (×63) and filters for GFP.

Statistical analysis. Differences between the groups in tumor metastasis, size and sample fluorescence were compared with Mann-Whitney and Student's *t*-test. Survival data of the mice was analysed with Cox–Mantel test and demonstrated as Kaplan–Meier analysis. Data are expressed as the mean±SD. Results at $p<0.05$ were considered statistically significant.

Results

DDGW therapy did not inhibit OCI-AML-3 or U-937 burden in vivo. The U937 or OCI-AML-3 tumours in mice treated with DDGW did not grow significantly more slowly compared to controls. At the last time point (day 11), when all the mice were alive, the tumors of DDGW-treated OCI-AML-3-bearing mice were even slightly larger 419±430 mm^3 compared to control mice 268±329 mm^3 ($p=0.27$); equally U937 tumors where 386±377 mm^3 in DDGW-treated mice whereas in control-treated mice they were only 294±340 mm^3 ($p=0.49$) (Figure 1). All tumours were limited to the subcutaneous space and no macroscopic invasion or clinical metastasis was detected.

DDGW therapy did not increase survival of OCI-AML-3 and U937-bearing mice. The DDGW therapy given did eventually seem to slow down the growth of OCI-AML-3 cells in mice, explaining why they later reached the euthanasia threshold, leading to a slightly longer overall survival (Figure 2A), however, the results were not statistically significant ($p=0.401$). For the U937 cell-bearing mice, the DDGW therapy had no effect at all on survival ($p=0.703$) (Figure 2B).

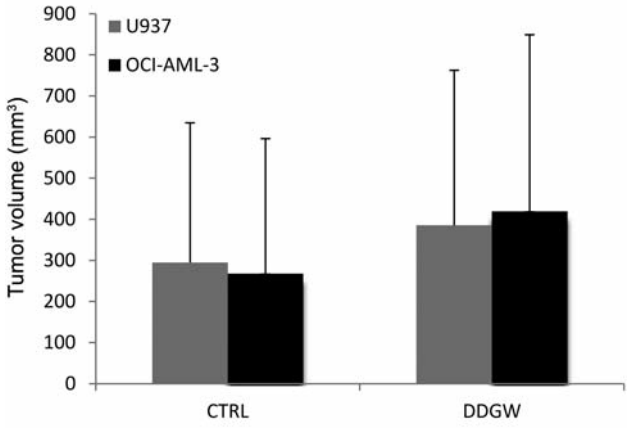

Figure 1. *DDGW therapy did not reduce OCI-AML-3 or U937 growth in vivo. DDGW did not linearly reduce the OCI-AML-3 and U937 xenograft burden of athymic mice. The last days when all the mice in the control (CTRL) and therapy (DDGW) groups were still alive the tumour volumes (mm^3) of the mice treated with DDGW were larger compared to those of the controls, but the effect was not statistically significant.*

LLG-GFP fusion did not target leukaemia tissue in vivo. The fluorescence of THP-1 tumour samples was quantified after injection of LLG conjugates. In general the visibility of leukaemia tissue in LLG-GFP fluoroscopy was slightly better compared to that in the controls, however, fluorescence of the tumour tissue was minimal in all groups compared to the surrounding stroma (Figure 3). There was no statistical difference between the accumulation of LLG-GFP and that of the controls Ala-GFP or His-GFP in tumor samples.

LLG-GFP fusion targets human inflammatory cells in vitro. Since it has been evident that LLG-GFP fusion is capable of targeting inflammatory cells in animal models and immortalized human cell lines (17, 18) we also tested its homing capacity on PDBu-activated human buffy coat. LLG-GFP attached to inflammatory cells during incubation as expected (Figure 4A), whereas controls His-GFP and Ala-GFP were washed away after incubation (Figure 4 B and C).

Discussion

The search for a new generation of immunomodulators as therapeutic tools has been intense. Several humanized antibodies are already in clinical use but their price and tendency to induce neutralizing antibodies in the host are well-known problems. For this reason, small molecular weight inhibitors are an active area of drug research. Peptide inhibitors LLG and DDGW developed against leukocyte $\alpha_M\beta_2$ Integrins are promising as they have been tested *in vitro* (9, 10), and they may have potential in the treatment of haematopoietic disorders and inflammatory conditions. Despite its narrow band target on the leukocyte surface, DDGW has been shown to be effective in prevention of inflammatory response *in vivo* (11). These results were later supported by experiments with a small molecule capable of super-activating $\alpha_M\beta_2$ Integrin: permanent β_2 activation arrests leukocytes onto their ligands and prevents their normal migration process, resulting in inhibition of the inflammatory response in a mouse model of peritonitis (12). This is an interesting finding since results of earlier studies on the role of β_2-Integrin subtypes in leukocyte activation and transmigration have been contradictory. In fact, the whole role of Mac-1 has sometimes even been questioned (21, 22). Several other strategies also focus on another subtype, LFA-1 (8, 23, 24). It seems that myeloid cells use LFA-1 as their cell surface receptor differently, depending on the tissue they migrate to (24), which considerably affects their use as therapeutic targets; whether this is the case with Mac-1 remains to be resolved. However, our previous results with non-peptide immunomodulators of Mac-1 demonstrated that targeting only a limited group of extracellular receptors can be sufficient to inhibit leukaemia and lymphoma growth *in vivo* (13).

Mac-1-targeting inhibitory peptides exerted some effects *in vivo* in a late-stage leukemia model (15), however, the 5-day therapy model used here, DDGW, clearly failed. This cannot be explained solely by the short therapy duration used since peptide-targeting matrix metalloproteinases (MMP) in an equivalent model with carcinoma cells was successful (20). It seems that MMPs may be very suitable for peptide-based intervention: MMP-targeting synthetic peptides have been successfully used in animal models to inhibit several malignancies such as sarcoma, melanoma and carcinoma (19, 20, 25, 26) which may reflect their potency. Fusion of MMP-targeting peptide to GFP has also been demonstrated to effectively target tumour tissue *in vivo* (19) in a model equivalent to that of the present study. The knowledge about the use of peptides such as LLG and DDGW as a therapy or diagnostic tool for malignancies is considerably less. Since β_2 Integrins are expressed almost exclusively by leukocytes this makes Mac-1-recognising LLG-GFP highly interesting as a diagnostic tool. Our finding, however, suggest that Mac-1-targeting peptide constructs are not sufficient for inhibiting leukaemia and lymphoma cell growth in mice. The biological activity *in vitro* has been great but biological activity is clearly compromised. One explanation may be reduced bioavailability and peptidolytic degradation of the compound in mice. However, our earlier results with peptide MMP inhibitors have proven that translation from *in vitro* to *in vivo* experiments using equivalent peptide concentrations can still be biologically-active (20). Interestingly, also the LLG-GFP failed to home to THP-1-formed tumors, although its MMP-targeting sibling was earlier proven to be biologically-active (19). Subcutaneously-inoculated leukaemia cell-formed

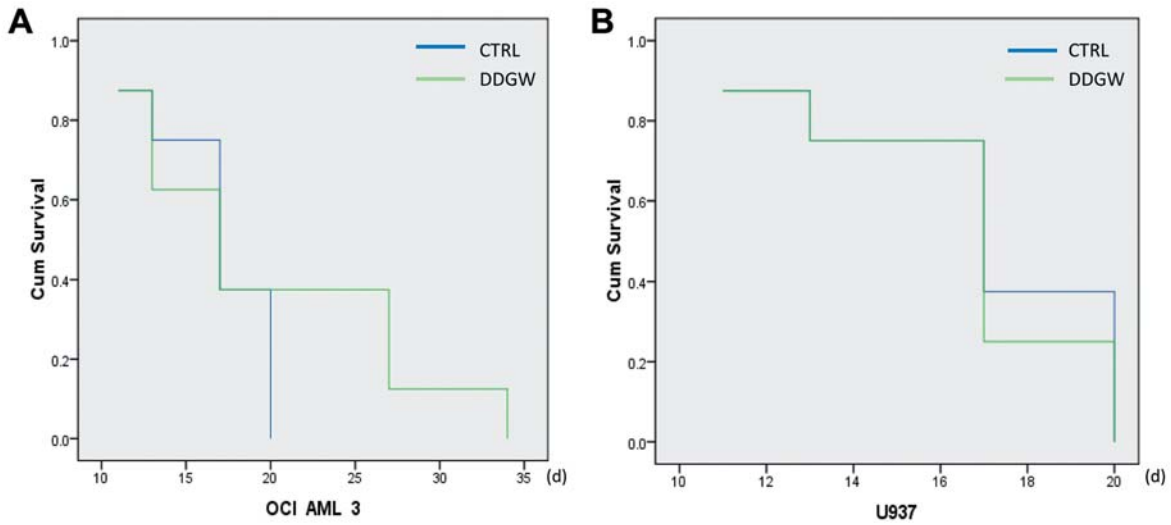

Figure 2. *DDGW therapy had no effect on mouse survival. The Athymic mice were allowed to live with their OCI-AML-3 and U937 xenografts while their well-being and xenograft growth were monitored as per the criteria for euthanasia. DDGW-treated OCI-AML-bearing mice survived slightly longer (A) as compared to their counterparts treated with control solution (CTRL) (n=8 in both groups). The differences detectable by Kaplan–Meier curves were not statistically significant. In the case of U937 cell-bearing mice (n=8) there was no difference at all as compared to the control (B). Abreviation (d) means the number of days from cell inoculation.*

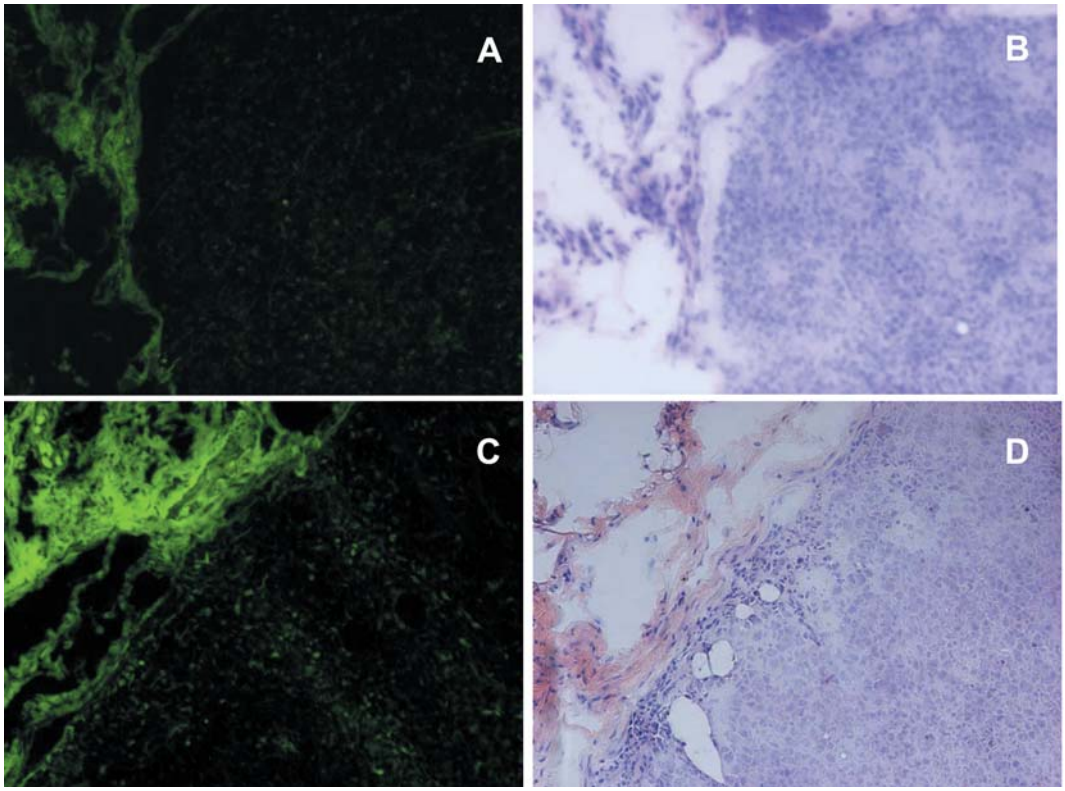

Figure 3. *CPCLLGCC-peptide – Green Fluorescent Protein fusion (LLG-GFP) did not home to xenografted THP-1 cells in vivo. Athymic mice were also inoculated with THP-1 leukemia cells expressing Mac-1 after which the xenografts were allowed to grow to be clinically visible (mean diameter 6 mm). The LLG-GFP fusion protein was injected into mice and after one hour tumour samples dissected from the euthanized mice. There was no statistically significant difference in the sample fluorescence between controls. The sample fluorescence of His-GFP which does not recognize Mac-1 (A) and LLG-GFP (C) was not significantly different. THP-1 containing areas of the resected tumours were identified from the haematoxylin-eosin-stained serial sections of the tumors; His-GFP (B) and LLG-GFP (D) which also demonstrates that fluorescence from THP-1cells is modest compared to stromal background fluorescence. Magnification, ×400.*

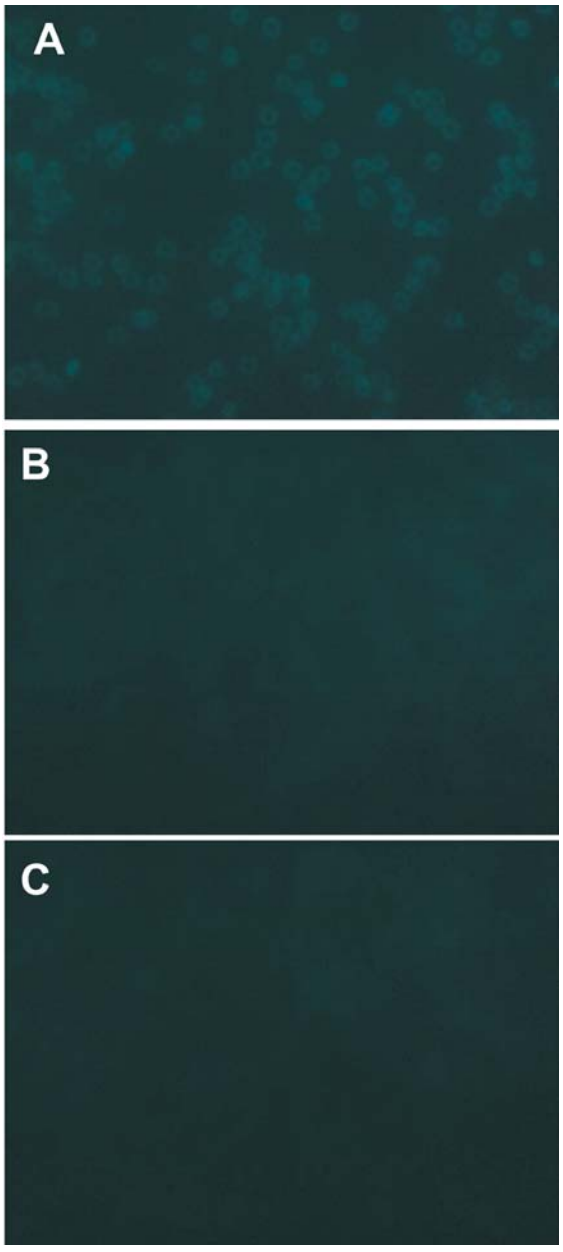

Figure 4. *CPCLLGCC-peptide – Green Fluorescent Protein fusion (LLG-GFP) targets activated human inflammatory cells. Activated buffy coat was incubated with LLG-GFP (A), His-GFP (B) and Ala-GFP (C). LLG-GFP treated buffy coat smear layer exhibited faint fluorescent signal, whereas controls had no signal over the background level. Magnification, ×63.*

tumours are, however, considerably less vascularised and do not fuse with underlying tissues like the carcinomas used previously; whether this explains the *in vivo* outcome seen here remains to be resolved. However, lack of peptide activity may be explained by the lack of bioavailability in target tissue rather than its poor bioactivity. Similar effects have been observed with lymphoid tissue zip code-targeting peptides, and for this reason, combination of *ex vivo* and *in vivo* panning techniques have been developed (27).

In summary, despite their clearly-proven ability to interfere both with inflammation *in vivo* and leukaemia cell motility *in vitro*, the peptides LLG and DDGW seem not to be sufficient at leukaemia or lymphoma targeting *in vivo*.

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