Volume 10, Number 1
January 1996

LEUKEMIA

Normal and Malignant Hemopoiesis

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Published on behalf of the Sven-Aage Killmann Leukemia Foundation

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Publisher: Jayne Marks
Production Controller: Sharon Clark
Supplement Editor: Alice Ellingham
Supplement Production Controller: Simone Larché

Subscriptions – EU/Rest of World Subscription price per annum (one volume, twelve issues); EU £475, rest of the world £475 (surface), £570 (airmail) or equivalent in any other currency. Personal subscriptions to be accompanied by a personal cheque or credit card details can be obtained for £199. Orders must be accompanied by remittance. Cheques should be made payable to *Macmillan Magazines* and sent to: The Subscription Department, Macmillan Press Ltd, Houndmills, Basingstoke, Hampshire RG21 2XS, UK. Where appropriate, subscribers may make payments into UK Post Office Giro Account No. 519 2455. Full details must accompany the payment. Subscribers from EU territories should add sales tax at the local rate.

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Leukemia (ISSN 0887-6924) is published monthly by Macmillan Press Ltd, c/o Mercury Airfreight International Ltd, 2323 Randolph Avenue, Avenel, NJ 07001, USA. Subscription price for institutions is $905 per annum. 2nd class postage is paid at Rahway NJ. Postmaster: send address corrections to Macmillan Press Ltd, c/o Mercury Airfreight International Ltd, 2323 Randolph Avenue, Avenel, NJ 07001

ISSN 0887-6924

Typeset by Photo-graphics, Honiton, Devon
Printed on acid-free paper, effective with Volume 8, Issue 1, 1994
Printed and bound in Great Britain by Latimer Trend & Company Ltd, Plymouth

Leukemia (1996) 10, 321–326

Antileukemic activity of recombinant humanized M195-gelonin immunotoxin in nude mice

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A leukemia-selective immunotoxin was constructed by linking recombinant gelonin (rGel), a single chain ribosome inhibitory protein, to recombinant humanized M195 antibody (HuM195), which recognizes the cell-surface protein designated CD33. CD33 is an antigen found on myeloid leukemia blasts as well as myeloid progenitor cells but it is not expressed in detectable amounts on the ultimate hematopoietic progenitor stem cell. Our previous studies indicated that a non-recombinant humanized immunotoxin displayed specific, potent toxicity towards CD33-positive cells but not to CD33-negative cells *in vitro*. In the current study, a recombinant humanized immunotoxin, HuM195-rGel, was evaluated *in vivo* in a nude mouse model of human myeloid leukemias. HuM195-rGel was found to target leukemia cells rapidly *in vivo* and was subsequently internalized into the cells. For trials *in vivo*, nude mice were injected (ip) with 10^7 log-phase HL60 human leukemia cells 10 days prior to the start of i.p. HuM195-rGel treatments. HuM195-rGel demonstrated significant tumor suppressive activity in this model. While all mice treated with either saline, rGel alone, or HuM195 plus unconjugated rGel (at 10 or 14 days after transplantation) had rapid tumor growth or early deaths, 50% of mice treated with HuM195-rGel failed to develop leukemic tumors for 5 months and the other 50% had significantly retarded tumor growth after treatment with HuM195-rGel. Mice treated at later times (28 days after transplantation of leukemia cells) also showed delayed leukemia cell growth, but no cures. These data show that HuM195-rGel can target leukemia cells *in vivo* and can result in pronounced anti-leukemic effects.

Keywords: myeloid leukemia; CD33; immunotoxin; gelonin; HuM195

Introduction

Immunotoxins (IT) are a class of proteins that consist of a monoclonal antibody (mAb) covalently linked or genetically fused to a cytotoxic molecule and are thus able to direct potent cytotoxic protein to particular cells.[1,2] Ribosome inhibitory proteins (RIPs) can be specifically targeted to certain tissues through chemical conjugation or genetic fusion with mAb and thereby acquire cell-specific cytotoxicity.[3,4]

Gelonin toxin originally isolated from the seeds of *Gelonium multiflorum* is a single polypeptide chain in a class of proteins designated type I RIPs. Unlike the type II RIPs, for example ricin, which is composed of a ricin A chain and a lectin-like B chain, gelonin has a relatively low native cytotoxic activity due to the lack of a lectin B chain, which can nonspecifically bind to cell membrane glycoproteins. Like all plant-derived RIPs, gelonin damages 28S rRNA through a glycosidase that cleaves the glycosidic bond at a unique adenine base in the rRNA, thereby inhibiting protein synthesis.[5,6] Recently, the native gelonin protein was sequenced, a synthetic gene encoding gelonin was sythesized, and biological-active recombinant gelonin (rGel) was synthesized in *E. coli*.[7]

Gelonin has several advantages for use in immunotoxin-therapy compared to other RIPs, including the lack of the B chain containing the galactose-specific lectin domain responsible for much non-specific binding and toxicity.[4,5] As a result, free gelonin is much less toxic to intact mammalian cells *in vitro* and *in vivo* than type II RIP. Despite this relative safety, in a cell-free rabbit reticulocyte translation assay, gelonin demonstrates nearly equal biological activity to heterodimeric toxins.[8,9] In addition, immunotoxins containing an A chain separated from a B chain are, in general, less potent on intact cells[10,11] and the larger heterodimers may also be more immunogenic than the single chain toxins. Therefore, gelonin may have properties advantageous for the generation of potent and specific immunoconjugates.[12]

CD33 is a useful target antigen for therapy of myelogenous leukemias, as it is expressed on the cell surface of greater than 80% of leukemia isolates from patients with myeloid leukemia with an average antigen density of 10 000 sites per cell.[13–16] However, CD33 is not found on tissues outside the hematopoietic system.[13] Its expression within the hematopoietic system is limited to early myeloid progenitor cells, monocytes and dendritic cells. Importantly, CD33 is not found on the ultimate hematopoietic progenitor stem cell, thus allowing in principle, selective elimination of leukemia cells and early progenitors while preserving capacity for long-term regeneration of marrow cells.[14,17,18]

HuM195, a humanized version of M195 constructed by genetically grafting the murine complementarity-determining region (CDR) to a human IgG1 framework and constant regions, is reactive with CD33.[19] The humanized antibodies may be advantageous due to reduced immunogenicity, higher avidity, and longer serum half lives.[19–22] In addition, rapid internalization occurs upon binding of mAb HuM195 to CD33 both *in vitro* and *in vivo*.[19,22,23] This suggests that HuM195 can be a suitable candidate for immunotoxin studies in humans.

We recently described a HuM195-gelonin immunotoxin.[12] HuM195-gelonin did not completely prevent hematopoietic reconstitution *in vitro* after treatment as evidenced by bone marrow colony experiments, but bone marrow treated with HuM195-gelonin demonstrated a log reduction of colony formation. This is expected since colony forming unit-granulocyte monocytes (CFU-GM) express CD33.[13,15,24]

Clinical trials using ^{131}I labeled M195 have demonstrated rapid and specific localization of antibody to tumor sites, saturation of all available CD33 antigen, followed by intracellular internalization.[22,23] Dose escalation studies using ^{131}I-M195 resulted in greater than 99% killing of leukemic blasts with negligible toxicity outside of the hematopoietic compartments.[25] However, due to the long range cytotoxicity of the conjugated nuclide (approximately 50 cell diameters) killing of normal bystander cells occurs as well, requiring bone mar-

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Received 9 August 1995; accepted 16 October 1995

row transplantation at high doses. We propose that use of an anti-CD33 immunotoxin may avoid this problem by targeting cells bearing the CD33 antigen. In this paper, we describe the activity of immunotoxins constructed by chemical conjugation of HuM195 with recombinant gelonin in a mouse model of HL60 leukemia.

Materials and methods

Animals

Six-week-old female outbred Swiss nu/nu mice were obtained from the colony at Sloan Kettering Institute. All bedding material was sterilized before use; the cages were covered with an air filter and maintained in isolation cabinets. Animal handling and experiments were performed in aseptic atmosphere using a laminar flow hood.

Cell lines

HL60 (acute myeloid leukemia, CD33 positive), RAJI and DAUDI (both B lineage Burkitt's lymphomas, CD33 negative) were maintained in culture using RPMI 1640 supplemented with 10% Serum Plus (JRH Biosciences, Lenexa, KS, USA) and 10% heat inactivated fetal calf serum (Intergen, Purchase, NY, USA).

Antibodies and radiolabeled antibodies

HuM195, a humanized IgG1 reactive with human CD33 antigen, and HuFd79, a genetically engineered human IgG1 construct reactive with a herpes simplex virus antigen not found on HL60 cells, were prepared as described.[19] Antibodies were trace labeled with ^{125}I (New England Nuclear, Boston, MA, USA) using the chloramine T method to a specific activity of 2–10 μCi/μg as described previously.[16] Immunoreactivity of the radiolabeled antibody was determined by incubating serial dilutions (10^7 to 10^6 cells) of HL60 cells with 2–4 ng of radiolabeled antibody for 1 h at 4°C. Cells were resuspended in phosphate buffered saline twice and the pellets were counted to determine total cell-bound ^{125}I-antibody.

Toxins

Recombinant gelonin (rGel) was derived and purified as described.[7] Functional activity studies demonstrated that this protein behaved similarly to chemically purified natural gelonin.[7]

Preparation of HuM195 Conjugates

HuM195 was conjugated with rGel using N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP); the immunoconjugates were purified by gel-permeation chromatography and separation on cibachron blue sepharose as previously described.[12] The molecular weight of HuM195-rGel was about 180 kDa demonstrating a 1 : 1 molar ratio of HuM195 to rGel.

Flow cytometry assays

Cells were washed and resuspended in 2% rabbit serum (Pel Freeze, Rogers, AK, USA) to reduce nonspecific binding. 5×10^5 cells in a final volume of 0.1 ml were incubated 1 h on ice in the presence of primary antibody HuFd79 control or HuM195. Cells were washed twice, incubated 30 min on ice with secondary fluorescein isothiocyanate (FITC) labeled antibody (goat anti-human immunoglobulin) (Kirkegaard and Perry, Gaithersburg, MD, USA), washed twice, and fixed with 0.5% paraformaldehyde. FITC fluorescence intensity was measured on an EPICS Profile II flow cytometer (Coulter, Miami, FL, USA).[15]

Inhibition of tritiated thymidine or leucine incorporation

An aliquot containing 100 μl of cells were washed and incubated at 37°C in 96-well plates in the presence of 50 μl of antibody, conjugate or toxin. After an incubation time of 3–7 days, 50 μl of 10 μCi/ml of tritiated thymidine or leucine (Du Pont-New England Nuclear) was added to each well and allowed to incorporate for 5 to 6 h. Trichloroacetic acid was added to a final concentration of 10% to precipitate protein for ^{3}H-leucine incorporation experiments. Cells were harvested using a semiautomatic harvester (Skatron) and read in a scintillation counter LS 6000IC (Beckman, Fullerton, CA, USA).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide : thiazolyl blue (MTT) assays

One hundred microliters of cells were washed and incubated at 37°C in 96-well plates in the presence of 50 μl of antibody, conjugate or toxin. After an incubation time of 3–7 days, the plate was centrifuged 5 min at 1000 r.p.m. MTT diluted in phosphate-buffered saline, was added to each well and incubated for 4 h. Plates were washed and the formazan product was solubilized with 0.04 M HCl in 2-propanol and quantitated spectrophotometrically at 570 nm.

Transplantation HL60 cells into nude mice

A 0.2-ml aliquot containing 10^7 HL60 cells from suspension culture was transplanted ip into nude mice. Tumors grew subcutaneously and the cutaneous tumor size was measured as a cross product to derive surface area. For studies *ex vivo*, pieces of tumor were minced and intact single cells were isolated on a Ficoll–Hypaque density gradient or after passage through a 70 μm nylon filter (Spectrum, Houston, TX, USA).

In vivo measurement of antibody targeting to tumor

The rate at which ^{125}I-HuM195 was bound to and subsequently internalized into leukemia cells *in vivo* was measured. Tumor bearing mice at 4 weeks (two mice per group) infused ip with 2 or 20 μg of ^{125}I-HuM195 or ^{125}I-HuFd79 (a negative control), were sacrificed at 4 or 24 h after the infusion. Tumors were excised and weighed at 4°C to avoid internalization during the assay process. Cell surface bound ^{125}I-HuM195 or ^{125}I-HuFd79 was then stripped using 50 mM

glycine/HCl, 150 mM NaCl, pH 2.8, and internalized c.p.m. (residual c.p.m. in the cell pellets) were calculated.[19] Specific surface bound and internalized HuM195 were calculated by subtraction of nonspecific surface bound and internalized HuFd79.

Immunotherapy

The test animals were treated i.p. with HuM195-rGel (36 μg/dose), recombinant gelonin (6 μg/dose), or HuM195 and recombinant gelonin mixture (30 μg HuM195 plus 6 μg gelonin/dose) twice a week in a final volume of 0.2 ml (the molar amounts of toxin and antibody were kept constant). Assuming a circulation volume of about 2 ml, the dose injected was equivalent to 100 nM initial concentration. Control mice were treated with 0.2 ml saline twice a week.

Results

In vitro cytotoxicity

HuM195-rGel was tested for its ability to kill CD33 positive and CD33 negative cells in comparison to free rGel. Activity and cytotoxicity were determined by inhibition of incorporation of ^{3}H-leucine into protein and by trypan blue exclusion. Dose–response curves were generated by testing the inhibitory effects HuM195-rGel on the protein synthesis of HL60 cells (CD33 positive) and RAJI (CD33 negative) in culture (Figure 1a). In the *in vitro* assays, the concentration of HuM195-rGel required to inhibit protein synthesis in HL60 cells by 50% was 0.6 nM, whereas the concentration of rGel alone required to nonspecifically inhibit protein synthesis in both HL60 and RAJI cells by 50% was about 200 nM (Figure 1a). In the concentration range of 10–100 nM HuM195-rGel, protein synthesis in HL60 cells was almost completely inhibited while no cytotoxicity was observed with the CD33 negative cell lines RAJI (Figure 1a) and DAUDI (not shown). However, HuM195 alone did not affect the protein synthesis in CD33 positive HL60 cells (Figure 1a). This shows that the inhibition of protein synthesis was due to specific binding and activity of the immunotoxin, and not a nonspecific property of the antibody itself. The specific targeting of leukemic cells by HuM195-Gel appeared to occur via the CD33 antigen binding site and not through the Fc region or other non-specific binding sites on target cells as shown previously.[12]

The cytotoxicity of HuM195-rGel was directly determined by trypan blue analysis. The concentration of HuM195-rGel required to kill 50% of cells was 0.7 nM (Figure 1b), similar to the concentration of HuM195 required to inhibit protein synthesis by 50% (Figure 1a). However, HuM195-rGel did not kill CD33 negative RAJI cells at the highest concentration of 100 nM, suggesting that it may be used safely for study *in vivo*. The cytotoxicity was also confirmed by ^{3}H-thymidine incorporation and MTT assays (not shown), confirming that HuM195-rGel causes HL60 leukemia cell death *in vitro*.

Targeting of radiolabeled HuM195 into leukemic cells in vivo

We have previously shown that nude mice retain limited ability to generate antibodies to the HL60 cells after transplant and that CD33 can be down-regulated by this response.[26]

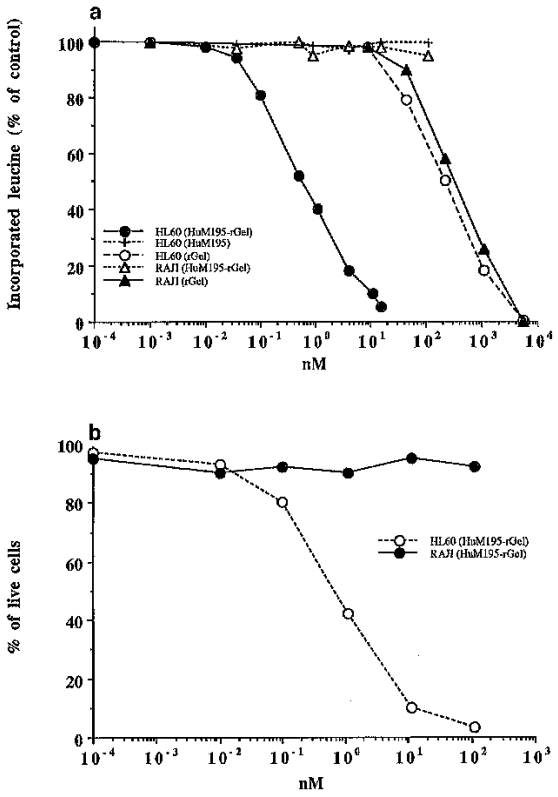

Figure 1 Cytotoxicity and inhibition of protein synthesis in HL60 or RAJI cells by recombinant gelonin (rGel), HuM195 and HuM195-rGel. (a) Inhibition of protein synthesis in HL60 or RAJI cells by rGel, HuM195 and HuM195-rGel. HL60 or RAJI cells at a final concentration of 10^5 cells/ml were incubated 3 days at 37°C in the presence of HuM195-rGel, HuM195 and rGel. Levels of protein synthesis were determined by 5-h incorporation of tritiated leucine into trichloroacetic acid precipitable protein. The treatment is shown in parenthesis. (b) Cell viability determined by trypan blue exclusion. HL60 or RAJI cells at a final concentration of 10^5 cells/ml were incubated 3 days at 37°C in the presence of HuM195-rGel. Trypan blue was added and live and dead cells were counted under the microscope.

Therefore, the expression of CD33 on the tumors was assessed. The cells from the leukemic tumors retained expression of CD33 positive antigen after growth *in vivo*, as determined by flow cytometry at saturating mAb concentrations (Figure 2). The internalization of ^{125}I-HuM195 into the target cells *in vivo* was rapid, and similar to the observations *in vitro*.[19] At 4 h after infusion of 2 or 20 μg antibody, 23–26% of bound ^{125}I-HuM195 was internalized, whereas a higher rate of internalization (38–43%) was seen at 24 h (Figure 3).

In vivo antitumor effects of HuM195-rGel

The leukemic cell growth in the subcutaneous space and peritoneum of nude mice was substantially reduced by HuM195-rGel. At 10 days after transplantation of HL60 cells into the peritoneum of nude mice, tumors of about 2 mm^2 in size are present in the subcutaneous space (Figure 4). After three injections of HuM195-rGel at a dose of 36 μg per mouse beginning at 10 days, two out of four mice did not develop tumors for

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