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PII S0024-3205(98)00352-X

Life Sciences, Vol. 63, No. 11, pp. 949-954, 1998

0024-3205/98 $19.00 + .00

THE Hsp90-SPECIFIC INHIBITOR, GELDANAMYCIN, BLOCKS CD28-MEDIATED ACTIVATION OF HUMAN T LYMPHOCYTES

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(Received in final form July 1, 1998)

Summary

The 90 kDa heat shock protein (Hsp90) is a molecular chaperone aiding the folding of nuclear hormone receptors and protein kinases. Hsp90-mediated folding can be disrupted by the Hsp90-specific drug, geldanamycin. Here we provide evidence for the inhibition of the CD28-specific BW 828 antibody-mediated activation of human T lymphocyte proliferation, IL-2 secretion and IL-2 receptor expression by geldanamycin. Our results suggest that the major cytoplasmic chaperone, Hsp90, plays an important role in CD28-mediated T lymphocyte activation.

Key Words: geldamycin, Hsp90, molecular chaperones, T lymphocyte activation, CD28, IL-2 secretion, IL-2 receptor expression

Molecular chaperones are highly conserved, ubiquitous mediators of the folding, assembly, transport and disposal of other proteins (1) and probably played a major role in the development of modern enzymes (2). The 90 kDa heat shock protein (Hsp90) is a major cytoplasmic chaperone helping the folding of nuclear hormone receptors and various protein kinases (3-6). Geldanamycin was originally developed as a tyrosine kinase inhibitor, but later it was proved to bind specifically to the non-canonical N-terminal ATP/ADP-binding site of Hsp90-homologues (7-9). Geldanamycin does not inhibit purified tyrosine kinases rather disrupts the folding and activation of several protein kinases, such as Raf and Lck inducing their degradation by the proteasome (4,5,10-12). A direct competition of geldanamycin with targets of Hsp90 has been recently demonstrated (13,14). Thus its "kinase-inhibition" is mediated by a more complex, folding-related mechanism.

T-cell receptor-mediated activation of T lymphocytes requires a co-stimulatory signal. This signal is usually provided by the CD28 receptor, which is necessary for the sustained response leading to cell proliferation (15). CD28 activation is mediated by an alternative MAPK (Rac/Jun kinase) pathway (16,17). Recently specific anti-CD28 monoclonal antibodies alone were shown to induce proliferation, IL-2 secretion and IL-2 receptor expression of human T lymphocytes (18).

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The present studies provide direct evidence for the presence of geldanamycin-sensitive steps in the alternative activation cascade of T lymphocytes mediated by CD28 receptors. Our results suggest the involvement of Hsp90 in these signalling processes.

Materials and methods

Chemicals -- The BW 828 IgG2a mouse monoclonal antibody (18,19) was from Behring (Marburg, Germany). For flow cytometric analysis of IL-2 receptor R-Phycoerythrin-conjugated anti-human CD25 mAb (mouse IgG1, PharMingen, San Diego, CA, USA) was used. Geldanamycin was from GIBCO-BRL (Gaithersburg, MD, USA). [^{3}H]-thymidine (20 Ci/mmol) was from Amersham Buchler GmbH (Braunschweig, Germany). Human IL-2 ELISA Kit was from Genzyme (West Malling, UK). All other chemicals used were from Sigma Chemicals Co. (St. Louis, MO, USA).

Cell preparation and stimulation of lymphocytes -- Human peripheral blood lymphocytes were isolated by Ficoll gradient centrifugation of heparinized buffy coat and depleted for contaminating nonadherent mononuclear cells by plastic adherence overnight at 37 °C as described earlier (20). Cells were cultured in RPMI 1640 medium supplemented with 10 % fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine at a density of 4 x 10^6 cells/ml. The viability of the cells was never less than 95 % as judged by trypan blue exclusion. To induce lymphocyte activation, cells (2 x 10^6/ml) were cultured in flat bottom microtiter plates at 37 °C and were stimulated with BW 828 (2.5 µg/ml), or PMA (1 ng/ml) and ionomycin (0.5 µg/ml) in the presence or absence of geldanamycin (1.78 µM) for the times indicated.

Cell-proliferation assay -- Control and stimulated lymphocytes were cultured in 96-well flat bottom microtiter plates at 5 x 10^4 cells/well in 200 µl at 37 °C. After 44 hours 0.5 µCi/well of [^{3}H]-thymidine was added for an additional 4 hours. Cells were harvested on glass-fiber filters using an automatic harvester and [^{3}H]-thymidine incorporation was determined by liquid scintillation counting.

IL-2 bioassay -- To determine IL-2 production, cells (10^6 in 500 µl) were cultured in flat bottom microtiter plates at 37 °C for 24 hours. The supernatants were harvested and the IL-2 concentration was measured using the commercial human IL-2 ELISA Kit obtained from Genzyme according to the manufacturers recommendations.

Analysis of IL-2 receptor expression -- Control and stimulated cells (2 x 10^6/1 ml) were incubated in a flat bottom microtiter plate at 37 °C. After 24 hours lymphocytes were resuspended at 5 x 10^5 cells/250 µl in blocking medium (PBS, 0.1 % BSA) and stained in round bottom microtiter plates with saturating amounts of R-phycoerythrin-conjugated anti-CD25 mAb for 15 min at room temperature in dark. Cells were then washed three times and resuspended in 250 µl of blocking buffer. Samples were analyzed in a FACScan flow cytometer (Becton-Dickinson, San Jose, CA, USA). The gating of lymphocytes was set on the basis of forward and side scattering characteristics of the cells.

Results

Geldanamycin blocks CD28-mediated T lymphocyte proliferation -- Confirming the earlier data of Siefken et al. (18) addition of the BW 828 CD28-specific antibody alone to peripheral blood T lymphocytes induced a strong proliferative response indicated by an 18-fold induction of thymidine uptake, which was 64 % of the stimulation achieved by a treatment with the simultaneous addition of the phorbol ester, PMA and ionomycin (Fig. 1). Geldanamycin reduced the thymidine uptake of stimulated T lymphocytes to the control level exhibiting a complete inhibition of the CD28-mediated proliferative response (Fig. 1). The effect of geldanamycin was not a consequence of a general cell toxicity induced by the drug, since both trypan blue exclusion studies and propidium-iodide FACS-analysis indicated a cell death less than 7 % after geldanamycin treatment (data not shown).

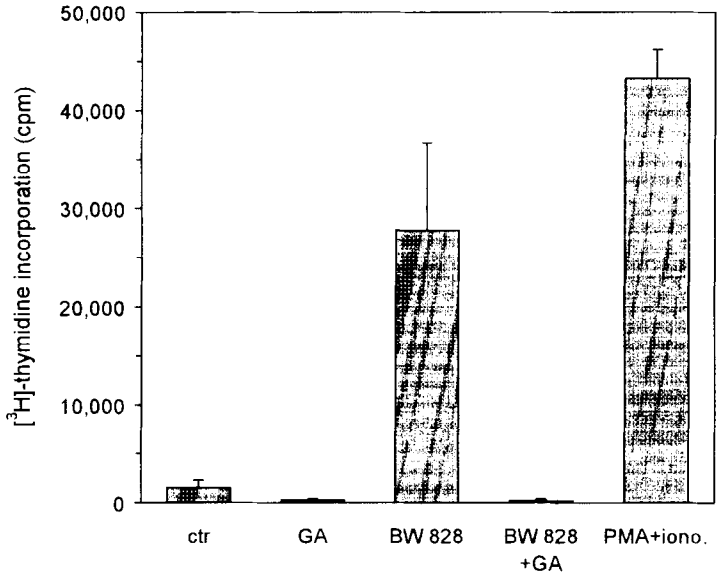

Fig. 1

Effect of geldanamycin on CD28-mediated proliferative response of T cells. Purified lymphocytes (2 x 10^6 cells/ml) from three different blood donors were treated with geldanamycin (GA), the CD28-specific antibody, BW 828, and PMA+ionomycin (PMA+iono.) at final concentrations of 1.78 µM, 2.5 µg/ml, 1 ng/ml and 0.5 µM, respectively. [^{3}H]-thymidine uptake was assessed as described in Materials and Methods. Results are expressed as means +/- SD of the three independent experiments done in triplicate cultures.

Geldanamycin inhibits CD28-induced IL-2 receptor expression -- CD28 stimulation of T lymphocytes leads to the expression of IL-2 receptors (18 and Fig. 2). Over 50 % of T cells expressed high affinity IL-2 receptors after anti-CD28 antibody treatment as compared to 60 % upon PMA+ionomycin addition (Fig. 2). Geldanamycin-treatment almost completely reversed the CD28-induced IL-2 expression to the control level (Fig. 2).

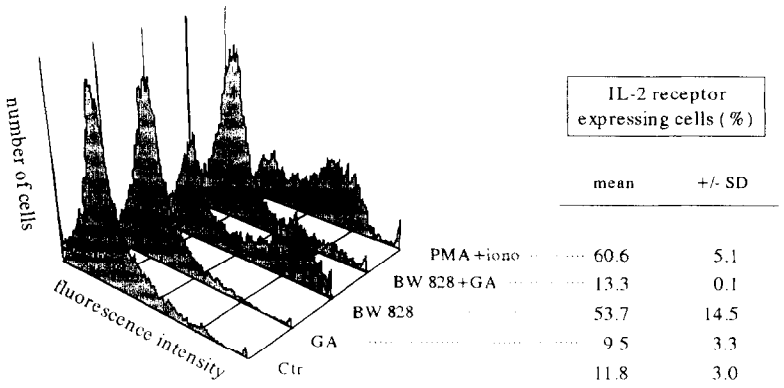

Fig. 2

Effect of geldanamycin on IL-2 receptor expression. Purified peripheral blood lymphocytes (2 x 10^6 cells/ml) from three different blood donors were treated with geldanamycin (GA), the CD28-specific antibody, BW 828 and PMA+ionomycin (PMA+iono.) at final concentrations of 1.78 μM, 2.5 μg/ml, 1 ng/ml and 0.5 μM, respectively. Expression of the alpha-chain of IL-2 receptor was measured by FACS analysis as described in Materials and Methods. The figure shows representatives of three FACS diagrams and the percent of IL-2 receptor expressing cells. Results are expressed as means +/− SD of three independent experiments.

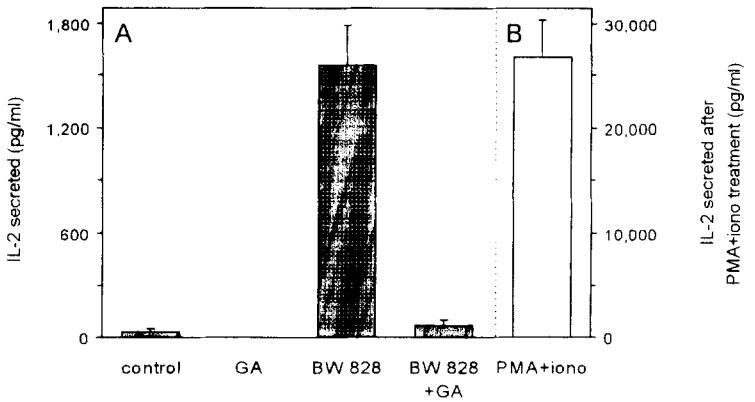

Fig. 3

Effect of geldanamycin on IL-2 secretion. Purified lymphocytes (2 x 10^6 cells/ml) from three different blood donors were treated with (A) geldanamycin (GA), the CD28-specific antibody, BW 828 and (B) PMA+ionomycin (PMA+iono.) at final concentrations of 1.78 μM, 2.5 μg/ml, 1 ng/ml and 0.5 μM, respectively. IL-2 secretion was determined by ELISA assay as described in Materials and Methods. Results are expressed as means +/− SD of three independent experiments.

Geldanamycin blocks CD28-dependent IL-2 secretion -- In agreement with earlier reports (18,21,22) activation of CD28 receptors induced a 52-fold increase in IL-2 secretion, which was 6 % of the stimulation after PMA+ionomycin treatment (Fig. 3). Geldanamycin caused a 22-fold reduction in the CD28-mediated IL-2 secretion (Fig. 3).

Discussion

It is well documented that the CD28 receptor can provide co-stimulatory signals complementing CD3/TCR-initiated cell proliferation. Stimulation of the CD28 molecule *alone* was regarded to induce no prolonged activation of T lymphocytes, or was reported even downregulate the T cell response (15,17,23). Recent data on human (18,21,22) and rat (24) T cells, however, provided several examples of CD28-mediated T lymphocyte activation. The CD28-specific antibody, BW 828, used in the present experiments binds exclusively to CD28 molecules and induces T-cell activation (18).

The Hsp90-specific drug, geldanamycin, blocks the CD28-induced cell proliferation and causes a significant inhibition of CD28-mediated IL-2 receptor expression and IL-2 secretion. Geldanamycin also introduces a block to the T-cell receptor-mediated lymphocyte activation which is most probably induced by the disruption of Hsp90-Lck and Hsp90-Raf complexes. However, geldanamycin neither has a general toxic effect, nor prevents the operation of all activation pathways since it does not affect the tyrosine phosphorylation of several molecules and does not block the elevation of intracellular calcium concentration of T cells (T. Schnaider, J. Somogyi, P Csermely, and M. Szamel, manuscript in preparation).

The CD28-specific antibody, BW 828, used in our experiments does not directly induce the traditional (Ras/Raf) MAPK pathway (18). On the contrary, similarly to the co-stimulatory functions of CD28 it activates the alternative MAPK (Rac/Jun kinase; 15,16) and the calcineurin/NF-AT pathways (18). Our results demonstrating the geldanamycin-induced inhibition of CD28 responses suggest the involvement of Hsp90 in the alternative MAPK pathway and/or in its cross-talk with the "traditional" MAPK pathway in CD28-mediated T lymphocyte activation.

Acknowledgments

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 265/A9), by research grants from the Volkswagen Foundation (I/73612), Hungarian Science Foundation (OTKA-T17720, T25206), Hungarian Ministry of Social Welfare (ETT-493/96), Hungarian Ministry of Culture and Education (FKFP 761/97), and from the ICGEB. T.S. acknowledges the support of the Soros Foundation and the Semmelweis University. P.C. is a recipient of an International Research Scholar's Award (HHMI 75195-541701) of the Howard Hughes Medical Institute. We would like to thank Mss. Marina Golombek and Annette Garbe (Div. Molecular Pharmacology, Medical School Hanover) for excellent technical assistance. T. S. is thankful to Dr. Reinhard Schwinzer (Div. Transplantation, Medical School Hanover) for helpful discussions.

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