Control of Apoptosis and Growth Factor's Signal in Glomerulonephritis

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Recently a great advance of intracellular signal study is accomplished with molecular biologic techniques. We are studying on intracellular signal mechanism of kidney diseases, especially, apoptosis in early stage of experimental glomerulonephritis and growth factors signal in late stage.

RATIONALE AND LIMITATION OF INTRACELLULAR SIGNAL STUDY

Current systemic immunosuppressive therapies, i.e. steroid therapy or cytotoxic agents, are associated with serious complications. During late decades there are numerous studies on multiple inflammatory or growth mediators, but those cytokines make too complicated network or are involved in too many biological responses of cells especially in vivo. To accomplish more specific control of the biologic responses, our study is focused on intracellular signal pathways. Distal signaling pathways may mediate specific cellular responses and certain signaling proteins may represent fundamental regulators of biologic response. But some limitation already exposed in this approach. There is redundancy of intracellular signal transduction pathways and heterogeneity of cellular lesions in experimental and human renal diseases. Moreover, the interruption of some signals are vital for function or survival of normal cells.

METHODS

1. Materials

Cell culture materials were purchased from Gibco (Grand Island, NY, USA). PDGF BB was obtained from Amgen (Thousand Oaks, CA, USA). PDGF receptor beta antibody was purchased from Upstate Biotechnology Inc. (Lake Placid, NY, USA) Protein A sepharose CL 4B was from Amersham Pharmacia (Piscataway, NJ, USA). All other reagents were of analytical grade.

2. Cell Culture

Isolation and characterization of normal rat or human mesangial cell were done as routine manner. Rat mesangial cells were grown in RPMI 1640 with 17% fetal calf serum and human mesangial cell were grown in DMEM also with 17% fetal calf serum. The passage numbers of rat mesangial cells were 17 to 35 and those of human mesangial cells were 8 to 15.

3. Cell proliferation assay

DNA synthesis in response to PDGF was measured as the amount of [methyl-^{3}H]-thymidine incorporated into trichloroacetic acid (TCA) precipitatable material. Cells were plated in 24-well dishes at a density of 5×10^4 cells/well and incubated with complete medium until

they become confluent. Quiescence was made with 48 hours serum free period, then stimulated for 20 hours by growth factors with/without inhibitors and pulsed for 4 hours with 1.0 μCi/ml [^{3}H]-thymidine. At the end of pulsing period, medium was carefully aspirated, ice-cold 5% TCA was added, and dishes were kept on ice for 5 min. After two additional washes with 5% TCA, cells were solubilized in 0.25 N NaOH-0.1% Sodium dodesyl sulfate solution. The incorporated radioactivity was measured with beta scintillation counter.

4. Anti- Thy 1 GN

Female Lewis rats weighing about 200 g were used for anti-Thy1 glomeruloneprhitis model. Anti-Thy 1 antibody (OX-7) was purchased from Cedarlane laboratory (Canada). The antibody was injected intravenously through tail vein at the dose of 250 μg/200 g of rat body weight. For apoptosis of early stage to GN study, the kidneys were harvested at 90 min after antibody injection. For tyrosine kinase inhibitor study, the PDGFR inhibitors (SU-6663 : 3 mg/200 g, SU9869 : 4 mg/200 g rat body weight) were injected at days 3, 5, 7, 9, intraperitoneally and the kidney tissue was obtained by surgically open biopsy or sacrifice at days 5, 7, 9, 14. For detection of proliferating cell, BrdU solution (5 mg/dl) was injected intraperitoneally 2 hours before biopsy or sacrifice. The tissues were snap frozen with liquid nitrogen or fixed in 10% phosphate buffered formalin for paraffin embedding or proceeded immediately to glomerular preparation with conventional sieving method.

5. PDGF receptor inhibitors

PDGF receptor tyrosine kinase inhibitors, SU6663 and SU9869, were developed at SUGEN (CA, USA). Molecular weight of SU6663 is 296, and SU9869 is 402. Both drugs were dissolved in dimethyl sulfoxide (DMSO).

6. Caspase Activity Assay & Caspase Inhibitors

ICE and Cpp32 activities in whole kidney tissue or prepared glomeruli were measured as described before (Nicholson et al.). Briefly, the kidney was homogenized glass homogenize tube and Teflon pestule in lysis buffer on ice slush. The lysis buffer contained 10 mM HEPES/KOH (pH 7.4, Gibco GBL), 2 mM EDTA, 0.1% CHAPS, 5mM dithiothreitol, 1 mM phenylmethyl-sulphonylfluoride (PMSF), 10 mg/ml pepstatin A, 20 mg/ml leupeptin, 10 mg/ml aprotinin (all above from Sigma). The lysate were centrifuged at 25,000 g for 10 min and the supernatant was incubated for one hour at 37℃ with 10 nmole of substrate (ICE substrate : Ac-YVAD-AMC, Cpp32 substrate : Ac-DEVD-AMC, Alexis corporation Inc.). Substrate cleavage was measured with spectrofluorometer (Fluoroskan Ⅱ) and were corrected as protein content in lysate (Bradford et al). ICE inhibitors (Ac-Tyr-Val-Ala-Asp-CHO : Ac-YVAD-CHO or Z-Val-Ala-Asp-(OMe)-fluoromethylketone : Z-VAD-(OMe)-FMK) and Cpp32 inhibitors (Ac-Asp-Glu-Val-Asp-CHO : Ac-DEVD-CHO) or Z-Asp-Glu-Val-Asp-(Ome)-fluoromethylketone : Z-DEVD-(Ome)-FMK) were used for apoptosis inhibition in glomerulonephritis *in vitro* and *in vivo*.

7. PDGF Receptor tyrosine kinase assay

PDGF receptor tyrosine kinase activity was measured directly on the immunobeads as described previously. Briefly, the cells or harvested kidney glomeruli were lysed in radio-immunoprecipitation assay (RIPA) buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1mM Na_3VO_4, 1% NP40, 1 mM PMSF and 0.1% aprotinin) at 4℃ for 30 min. Cleared lysate was prepared by centrifugation. Protein

amount was estimated in the cleared supernatant, and equal amount of lysate protein was incubated with the PDGFR β-antibody on ice for 30 minutes. Protein A sepharose beads were added and the tubes were rotated at 4℃ for two hours. The beads were washed twice with RIPA buffer, twice with washing buffer (50 mM Tris-HCl pH 7.4, 1 mM Na_3VO_4) and finally resuspended in 2X reaction buffer. The autophosphorylation reaction was performed with [γ-^{32}P] ATP at 30℃ for 15 min. The reaction was stopped by cold RIPA buffer, and the beads were washed twice again with washing buffer. Boiled immunoprecipitates were separated on 7.5% SDS-polyacrylamide gel.

8. PI 3 Kinase assay

The lysate of cell or glomeruli was prepared as above tyrosine kinase assay. The immunobeads were resuspended with PI 3 Kinase assay buffer (20 mM Tris-HCl pH 7.5, 0.1 M NaCl, and 0.5 mM ethylene glycol-bis (β-aminoethyl ether)-N, N, N', N'-tetraacetic acid), 0.5 μl of phosphatidylinositol was added and incubated at 25℃ for 10 min. One microliter of 1 M $MgCl_2$ and 10 μCi of [γ-^{32}P] ATP were added simultaneously to the reaction mixture and incubated at 25℃ for another 10 minutes. A mixture of chloroform-methanol and 11.6 N HCl (150 μl, at a ratio of 50 : 100 : 1) was added to stop the reaction. The reaction was then extracted with 100 μl of chloroform. The organic layer is washed with 80 μl of methanol and 1 N HCl (1 : 1). The reaction product was dried under a stream of nitrogen and resuspended in 10 μl of chloroform, separated by thin-layer chromatography, and developed with $CHCl_3$/methanol/28% NH_4OH/H_20 (129 : 114 : 15 : 21). The spots are visualized by autoradiography.

9. Immunohistochemical stain

Formalin fixed-paraffin embedded tissue sections were stained with hematoxylin and eosin. Frozen tissues were stored at -70℃ until use. 4 μm tissue cut was used for immuno-histochemical stain for Fas, FADD, Bax, Bcl-2, ICE, Cpp32 (Santa Cruz biotech, CA, USA), mitogen activated protein kinase (MAPK : Extracellular regulate kinase; ERK1, Santa Cruz biotech, CA, USA), monocyte-macrophage (ED 1, from DAKO, UK) and PDGFR (Upstate Biotech Inc., NY, USA). For detection of proliferating cells, incorporated BrdU staining was performed with anti-BrdU antibody (Boehringer Mannheim, Germany) after tissue permeabilization with triton-X. The primary antibody binding was visualized with avidin-biotin immunoperoxidase method and/or alkaline phosphatase (APAAP) technique. For quantitative analysis of total glomeular cells, the numbers of nuclei in 20 randomly selected mid-cut glomeruli in each tissue section were counted by two independent personnel.

RESULT ABSTRACTS

1. Apoptosis Regulatory Proteins In Immunecomplex-Mediated glomerulonephritis In The Rat : Role Of Complement

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Immunecomplex-mediated diseases results in cell injury that ranges from mild membrane perturbation to apoptosis and cellular necrosis. Anti Thy 1 antibody when injected into rats induces glomerular cell injury followed by cell proliferation. We investigated the expression of

proteins known to regulate apoptosis by immunohistochemistry in rats with anti-Thyl nephritis. Rats develop mesangiolysis within 24 hours after the injection, modest cellular repopulation at day 3 with prominent hypercellularity at day 7 which subsides by day 14 and day 28. Within 90 minutes after the injection of anti-Thyl antibody, approximately 10% of glomerular cells show apoptosis by TUNEL technique. Glomerular expression of proapoptotic proteins interleukin converting enzyme (ICE), Cpp32, Bax and FADD is increased. The expression of Bcl-2, a protein that protects against apoptosis is also increased. Double immuno-histochemical staining utilizing ED1 as monocyte/macrophage marker indicate that the expression of these proteins is predominantly by intrinsic glomerular cells. Very few (<2%) apoptotic cells are observed at day 7, 14, and 28. Complement depletion by intraperitoneal administration of cobra venom factor abolishes the expression of apoptosis regulatory proteins, as well as apoptotic cells by TUNEL technique. Macrophage infiltration does not change.

These data indicate a biphasic role of apoptosis in glomerular injury. An early phase that may contribute to cell injury and hypocellularity and a late phase that contributes to resolution of proliferative glomerulonephritis. Moreover, complement activation in this model may lead not only to cell lysis but also to apoptosis.

2. Blockade of PDGF Receptor Intracellular Signal Ameliorates Mesangial Proliferation in Immune Mediated Glomerulonephritis.

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In mesangial proliferative nephritis, platelet-derived growth factor (PDGF) is known as one of the strongest mitogen and migration stimulator after glomerular injury. PDGF exerts its effects on cells via binding to structurally similar α- and β- tyrosine kinase receptors. Recently, a new class of protein tyrosine kinase inhibitors was identified that is based on and oxindole core (indolinones). To explore the effect of PDGF receptor signal blockade on the mesangial cell over-proliferation, we applied PDGF receptor specific tyrosine kinase inhibitor to cultured mesangial cells and anti-Thyl glomerulonephritis. PDGF receptor tyrosine kinase inhibitors, SU6663 and SU9869, were administrated to cultured rat and human mesangial cells and anti- Thy 1 glomerulonephritis rat. In vitro, PDGF-induced tyrosine autophosphorylation of PDGF receptor and PI 3 kinase activity was measured with immuno-precipitation method, and DNA synthesis also measured by tritium thymidine incorporation. In vivo, anti-Thy 1 glomerulonephritis was induced by OX-7 antibody and mesangial cell proliferation was evaluated histologically. The both PDGF receptor inhibitors block the PDGF stimulated autophosphorylation of PDGF receptor beta and PI 3 kinase activity of the cultured rat and human mesangial cells as dose-dependent manner. PDGF induced DNA synthesis also completely blocked at the dose of 0.1 μM. Over proliferation of mesangial cells (total glomerular cell count on day 9) in anti-Thyl glomerulonephritis decreased 33.3% to 18.4% by the thrice injections of inhibitor (SU6663, 3 mg/200 g of rat body weight). Proliferating cell count (BrdU incorporated cell) also decreased 3.55 to 2.90 per glomerulus on day 7. PDGF receptor tyrosine kinase inhibition can block intracellular signal of PDGF for DNA synthesis in cultured mesangial cells and can ameliorate the over proliferation of glomerular cells in immune mediated mesangial proliferatiev glomerulonephritis.

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