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ROLE OF CD44 IN CTL AND NK CELL ACTIVITY

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1. ABSTRACT

CD44 is a widely distributed cell surface glycoprotein whose principal ligand has been identified as hyaluronic acid (HA), a major component of the extracellular matrix (ECM). Recent studies have demonstrated that activation through CD44 leads to induction of effector function in T cells and macrophages. At sites of chronic inflammation as seen in certain infections, autoimmune diseases, allograft rejection, graft-versus-host (GVH) disease and treatment of cancer patients with high doses of interleukin-2, significant damage to the endothelial cells has been known to occur, which leads to the toxicity or pathogenesis associated with the disease. The exact mechanism of endothelial cell damage is not clear, although, it has been widely speculated that immune cells may play a critical role. Studies from our laboratory have used interleukin-2 (IL-2) induced vascular leak syndrome (VLS) as a model to investigate the role of cytolytic lymphocytes in the direct cytotoxicity of endothelial cells. Cytotoxic T lymphocytes (CTL), double-negative (DN) T cells and natural killer (NK) cells upon activation express high levels of CD44 and mediate efficient MHC-unrestricted TCR-independent lysis following ligation of CD44. Such CD44-mediated cytotoxicity may play an important role in protection against viral infections and cancer. However, it could also cause non-specific tissue injury. For example, dysregulation in the interaction between activated cytotoxic lymphocytes expressing CD44 and endothelial cells bearing the appropriate ligand such as the hyaluronate (HA), could lead to endothelial cell lysis. Furthermore, such endothelial cell injury could lead to the pathogenesis associated with a variety of clinical diseases.

2. INTRODUCTION

It is well established that activation of T or B lymphocytes through their antigen-specific receptors leads to their differentiation and growth. In addition to the antigen-specific receptors, the T and B cells also express a variety of adhesion molecules, which are known to participate in cell-cell interaction, migration, homing and signal transduction. Recent studies have shown that the function of an adhesion receptor cannot be determined from its expression alone. Adhesion receptors are thought to be "selected" to perform distinct effector functions based on their cell-background and factors present in the local environment (1). Therefore, adhesion receptors that are expressed on different cell types may be in different states of "activation-readiness" and may continue to be selected by conditions in the surrounding environment to bind to tissue-specific ligands and mediate leukocyte effector functions (1). There has been a growing interest in lymphocyte interactions with extracellular matrix (ECM) components. Interaction of lymphocyte adhesion receptors with ECM has been shown to play a central role in regulating the migration, differentiation, and functions of the cells of the immune system (2). The interplay between the cell-surface and matrix molecules that regulate adherence, and chemoattractant gradients that direct cell migration, control the localization of cells (3). Thus the cells of the immune system depend on regulated interactions with other cells to activate and direct the response to infection (3). Recently, studies involving the interactions of T lymphocytes with antigen-bearing cells have exposed both antigen-specific receptors and a group of cell adhesion molecules. We have made an exciting observation that activated cytotoxic T lymphocytes (CTL), natural killer (NK)/lymphokine activated killer (LAK) cells, and cytotoxic double-negative (DN) T cells that accumulate in lpr/lpr mice, express high levels of CD44 and when activated through CD44 mediate efficient lysis of target cells (4-8). This observation was confirmed and extended by a number of investigators (9-11). Inasmuch as, CD44 also plays a major role in the lymphocyte

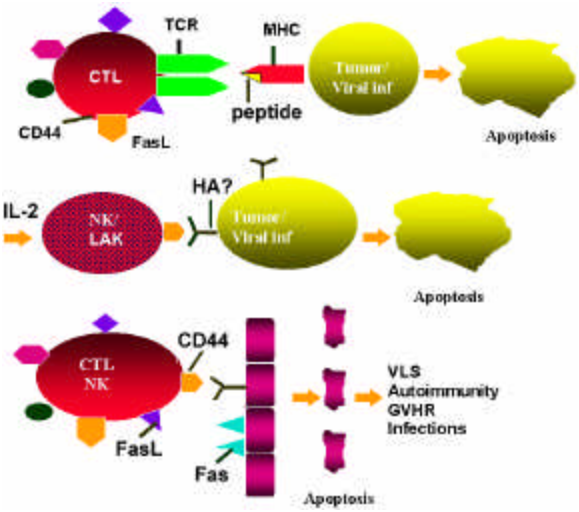

Figure 1. When CTL recognize a foreign peptide in association with class I MHC, when NK cells recognize similar cells or when CTL/NK cells are activated with IL-2, they express high levels of CD44 and cytolytic effector molecules (perforin and Fas ligand). This results in the killing of the target cells. The activated CTL/NK cells in certain instances can also cause lysis of endothelial cells. Such lysis is mediated via CD44-HA interactions involving Fas ligand and perforin. Such non-specific cytotoxicity of endothelial cells may account for vascular injury seen during VLS, autoimmunity, GVHR and infections.

adhesion to the endothelial cells, we hypothesized that dysregulation in the interaction between activated cytotoxic lymphocytes expressing CD44 and endothelial cells bearing the appropriate ligand such as the hyaluronate (HA), could lead to endothelial cell lysis. Furthermore, we suggest that such endothelial cell injury could lead to the pathogenesis associated with a variety of clinical diseases (figure 1).

3. ADHESION MOLECULES

Adhesion molecules play an important role in cell-cell and cell-extracellular matrix interactions (12). Such interactions are crucial to all developmental processes (13). Adhesion receptors of the immune system are important in regulating the mechanism of cell adhesion. In vivo, adhesion molecules guide cell interactions. The size and shape of adhesion molecules involved with the immune system have been determined by electron microscopy or X-ray crystallography. The distance between two cell membranes or a cell membrane and the extracellular matrix can be determined because the binding sites for several adhesion receptors and their counter-receptors are known (3). Lymphocyte adhesion receptors regulate lymphocyte antigen-specific interactions and also transmit information that affects cellular differentiation and responsiveness and interactions with the environment. There are important interactions between adhesion molecules and antigen receptors involving signaling and induction of gene expression. Adhesive interactions take place when lymphocytes have been activated by foreign antigen. These interactions will direct their localization and migration before receptors determine lymphocyte homing to different lymphoid organs and neutrophil localization in inflammation. Three families of adhesion receptors control these interactions: the immunoglobulin superfamily (includes the antigen-specific receptors of T and B lymphocytes), the integrin family (is important in dynamic regulation of adhesion and migration), and the selectins (are prominent in lymphocyte and neutrophil interaction with vascular endothelium) (3).

4. SIGNIFICANCE OF CD44

CD44 (also known as Pgp-1, Ly-24, extracellular matrix receptor III and Hermes) is synthesized as a 37 kDa molecule (14). CD44 is an acidic, sulfated integral membrane glycoprotein ranging in molecular weight from 80 kD up to 200 kD (15). The gene for CD44 has recently been cloned. The sequence has revealed that the CD44 protein backbone is a 37 kDa molecule that is extensively glycosylated via N- and O-linkages, and is rich in serine and threonine residues (22%) (14). Both physically and functionally, the CD44 molecule can be separated into three main regions: the cytoplasmic domain (mediates the interaction with the cytoskeleton), the middle domain (responsible for the lymphocyte homing) and the amino-terminal domain (which binds to HA) (16). The amino terminal portions of CD44 are homologous to cartilage link proteins, which promote proteoglycan- and collagen-dependent extracellular matrix adhesion. CD44 has also been shown to bind to extracellular matrix components such as hyaluronate, collagen, and fibronectin (17). Hyaluronate (HA) is a common component of the extracellular matrix and extracellular fluid (18). The CD44 family belongs to a larger group of HA-binding proteins, called the hyaladherins. CD44 is thought to function by mediating cell-cell or cell-substrate interactions through recognition of components of the ECM, intercellular milieu, and/or pericellular layer (18). CD44 ligand-binding function on lymphocytes is strictly regulated, such that most CD44-expressing cells do not constitutively bind ligand (18). There is not a one-to-one correspondence between the expression of CD44 on the cell surface and the ability to bind HA. CD44 ligand-binding functions may be activated due to differentiation, inside-out signaling, and/or extracellular stimuli (18). The affinity for hyaluronate can be experimentally controlled and depends on the cytoplasmic domain of CD44 (19)

CD44 is a diverse family of molecules produced by alternate splicing of multiple exons of a single gene and by different posttranslational modifications in different cell types (18). The influence of these modifications on ligand-binding are not fully understood and are still being studied. In mature lymphocytes, CD44 is upregulated in response to antigenic stimuli and may participate in the effector stage of immunological responses (18). Examination of the cDNA sequence of CD44 which showed homology between the amino-terminal portion of CD44 to chick and rat cartilage link proteins has provided evidence that CD44 has an ECM ligand. Subsequent studies have shown that HA is a ligand for CD44 (20). It has been reported that lymphoid cell lines (18), B cell hybridomas (21), and IL 5-activated B cells (22) have all been shown to bind to purified HA and the binding can be specifically inhibited by anti-CD44 mAbs. More importantly it has been shown that the CD44 expressed on the B cell hybridoma is involved in binding to HA present on the surface of the

stromal cells *in vitro* (23). These results along with earlier data showing inhibition by anti-CD44 mAb's of B cell lymphopoiesis in long-term bone marrow cultures (23) suggests that the CD44-HA interaction may be important in B cell differentiation.

CD44 is expressed by various lymphoid and nonlymphoid tissues (15, 24) and has been demonstrated to participate in lymphocyte adhesion to the matrix, lymph node homing and lymphopoeisis (15, 23). Recent studies have demonstrated that the CD44 molecule may also participate in lymphocyte activation. Studies from our lab demonstrated that antibodies against CD44 can trigger the lytic activity of the cytotoxic T lymphocytes (CTL) as well as the double-negative T cells that accumulate in the MRL lpr/lpr mice (4, 7). We have also shown that the cytotoxicity induced by T cells can be inhibited in the presence of soluble HA thereby suggesting that HA may serve as an important molecule involved in the target cell recognition by the cytotoxic T lymphocytes (unpublished observation). Similarly, monoclonal antibodies (mAbs) directed against CD44 molecules have been shown to either upregulate (25, 26) or downregulate (27) anti-CD3 and anti-CD2 mAb induced proliferation of T cells. Furthermore, certain anti-CD44 mAbs have also been shown to induce proliferation of resting human T cells in the absence of costimulation with anti-CD3 or anti-CD2 mAbs (9, 28, 29). All of these data together demonstrate that activation via CD44 can trigger effector functions in human T lymphocytes. In addition, antibodies against CD44 have also been shown to activate human monocytes and enhance the natural killer (NK) cell mediated cytotoxicity (11, 30).

5. ARE CYTOTOXIC LYMPHOCYTES INVOLVED IN NONSPECIFIC VASCULAR TISSUE INJURY SEEN *IN VIVO*?

There are many disease models in which factors other than cytolytic lymphocytes have been shown to participate in endothelial cell injury, such as, neutrophils, complement components, etc. However, there is growing evidence for the involvement of cytolytic lymphocytes in endothelial cell injury. For example, IL-2 activated T cells and other leukocytes have been shown kill endothelial cells ex vivo and in vivo (31-36). There are several unexplained disease models in which endothelial cell damage has been reported and it is tempting to speculate that such instances, damage can result from promiscuous killing exhibited by cytotoxic cells, using CD44 receptor.

Endothelial cell injury is one of the most widely noted phenomenons in a variety of clinical diseases. Murine lymphocytic choriomeningitis (LCM) viral infection represents a well-characterized experimental infection where massive delayed-type hypersensitivity (DTH) reaction occurs in the CSF, caused by $CD8^+$ T cells (37). The induction of inflammation and clinical symptoms of the disease can be prevented by immunosuppression, specifically inhibition of T cell responsiveness. It has been speculated that virally activated $CD8^+$ T cell which express high levels of CD44, kill endothelial cells leading to massive extravasation of monocytes and $CD4^+$ T cells in the subarachnoid space. In experimental allergic encephalomyelitis, a model for human multiple sclerosis, damage to the blood-brain barrier has been reported to occur, following injury to the endothelial cells, the exact mechanism of which is not known. This facilitates infiltration of $CD4^+$ T cells into the CNS. CD44-HA interactions have been shown to mediate lymphocyte adhesion to the white matter thereby contributing towards the pathogenesis of inflammation of CNS (38). Moreover, in autoimmune disease models involving vasculitides, the lesions have been associated with infiltration of lymphocytes and macrophages at the vascular wall structure (39). Such types of vasculitis have been described in human autoimmune disease (40) as well as in the mouse models (41). Also, lymphocytes activated with cultured endothelial cells have been shown to induce experimental autoimmune type of vasculitis (42). The T cell involvement in the endothelial cell damage leading to vascular disease in scleroderma has also been described (43). The peripheral blood lymphocytes from some patients with rheumatoid arthritis and giant cell arthritidis have been shown to be cytotoxic to endothelial cells but not to fibroblasts (44). Similarly, in arteriosclerosis, endothelial cell damage and inflammatory cell activation have been shown to contribute to the further development of cardiovascular disease (45, 46). The endothelial cells lining the inflamed vessels have been shown to play an important role in initiating chronic rejection of the graft, by the NK cells (47). Accelerated arteriosclerosis is a major complication in cardiac transplantation, caused in part by the rejection related cell-mediated immune response. Furthermore, examination of coronary arteries have revealed the presence of CTL expressing perforin (48). Donor heart endothelial cells have been shown to act as targets for infiltrating lymphocytes of the host after clinical cardiac transplantation (47, 49). Acute lethal GVH reaction can be induced using the T cells that can trigger VLS causing death of the recipient (50). All such examples stress the possible participation of cytolytic lymphocytes in endothelial cell damage.

5.1. Is CD44 Involved in the Induction of Vascular Leak Syndrome (VLS)?

At sites of chronic inflammation as seen in certain infections, autoimmune diseases, allograft refection, GVH disease and treatment of cancer patients with high doses of IL-2, significant damage to the endothelial cells has been noted, although the mechanism of such endothelial cell damage is not understood. The destruction of endothelial cells leads to loss of vascular fluid, edema in various organs and often death. This pathological condition is called vascular leak syndrome (VLS). VLS is seen only in immunocompetent but not in nude or immunodeficient mice receiving IL-2 (51, 52). Also, the toxicity associated with IL-2 therapy has been shown to decrease after depletion of NK/LAK cells *in vivo* (53).

The role of CTL in VLS induction was demonstrated in our earlier studies in which it was noted that administration of a CTL clone plus IL-2 into irradiated syngeneic mice but not the CTL clone or IL-2 alone, triggered VLS (32). Immunotherapy using such cells causes damage to the vascular endothelial cells and leads to pulmonary edema and respiratory problems. Also, IL-2 activated CTL clone could mediate efficient lysis of an endothelial cell line but not a fibroblast cell line, in a MHC-unrestricted fashion (32). Such studies together with the data presented in the current study suggest that IL-2 induced VLS may result from the direct cytotoxicity of endothelial cells by LAK cells. We and others have shown earlier that CTL, double-negative T cells and NK cells upon

activation express high levels of CD44 and mediate efficient MHC-unrestricted TCR-independent lysis following ligation of CD44 (7, 9, 11, 32, 54, 55). We have also demonstrated that the lysis of endothelial cells *ex vivo* by cytolytic lymphocytes can be blocked by soluble CD44 fusion protein, anti-CD44 Fab fragments or soluble hyaluronate (unpublished data). These data suggested that CD44-hyaluronate interactions may play an important role in the migration, homing and lysis of endothelial cells.

We have proposed that VLS results from the interaction between CD44 expressed on cytotoxic T cells and LAK cells and hyaluronate expressed on endothelial cells. Studies carried out in our lab show that perforin KO mice had decreased levels of VLS in the lung, liver and spleen and *gld* mice had decreased levels of VLS in the liver and spleen (66). This demonstrates for the first time that these cytolytic/apoptotic molecules are directly involved in vascular leak. This also suggested that VLS may result from active damage to the endothelial cells. Future studies will investigate whether preventing the interaction between CD44 and its ligand on the endothelial cells will inhibit the damage to these endothelial cells. The results of these studies may offer a treatment to a range of diseases where endothelial cell damage occurs.

5.2. TCR-independent, MHC-unrestricted killing and its significance

It has been shown that long-term culture of Ag-specific CTL clones or culture with high doses of IL-2, leads to "promiscuous" or MHC-unrestricted killing of Ag-negative target cells, similar to the killing exhibited by NK cells. Furthermore, culture of NK cells or naive T cells with concentrations of IL-2, also triggers MHC-unrestricted lysis often designated as LAK activity (31, 33, 56). The significance of such promiscuous killing by T cells is not clear. While TCR may help in the clonal expansion and lysis of MHC-bearing target cells, NK-like function can help in killing transformed cells or virally-infected cells which may escape TCR-directed recognition, by mechanisms such as, downregulation of MHC molecules (57). Several adhesion molecules involved in MHC-unrestricted killing have been characterized (58-61). In our lab, we have demonstrated that CD44 can serve as a key cytolytic effector molecule in both CTL and NK/LAK cells.

5.3. Role of CD44 and various isoforms in T cell activation and MHC-unrestricted lysis

CD44 is a broadly distributed family of cell surface glycoproteins, all encoded by a single gene. CD44 has been shown to play a major role in homing and cell adhesion. CD44 may also play an important role in signal transduction and activation of B and T cells (15, 26, 62, 63) and trigger monocytes to produce cytokines (64). CD44 is encoded by 20 exons, seven of which form the invariant extracellular region of the so called standard form (CD44s). By alternative splicing, up to 10 variant exons (CD44v1-v10) can be inserted within the extracellular region (19). Different CD44 isoforms expressed by the cell may affect the cell function. Thus far the role of different isoforms has been studied mainly in tumors. It was shown that isoforms CD44v6 and CD44v5 contribute to tumor progression and metastasis (18), whereas CD44 molecules containing the v3 exon bind certain growth factors and are not involved in the cell adhesion (18). Activation of normal lymphocytes correlates with the presence of splice variants (19). Current studies in our lab are testing whether different CD44 isoforms are involved in adhesion/homing and cytotoxcity by CTL/LAK cells.

6. CONCLUSIONS

In summary, CD44 is an important molecule involved in the activation of CTL/NK cells leading to the lysis of the target cells. CD44 is also used by lymphocytes to migrate and home to different organs or sites of inflammation. Inasmuch as endothelial cells bear the ligand for CD44, it is important to investigate the interaction between cytolytic lymphocytes and endothelial cells because in a number of disease conditions, endothelial cell injury has been shown to be a major contributing factor towards the pathogenesis. There is significant evidence to suggest the involvement of cytotoxic lymphocytes in the direct damage of the endothelial cells. Such endothelial cell injury may result from dysregulated interaction between CD44+ cytolytic lymphocytes and endothelial cells bearing the ligand for CD44. The TCR-independent CD44-mediated cytotoxicity by CTL/NK cells may represent a double-edge sword. On the one hand, it may play a beneficial role by killing virally infected or cancer cells that downregulate MHC or resist TCR-mediated lysis. On the other hand, such non-specific killing may account for tissue injury particularly at sites of chronic inflammation.

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