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CD40 Expression in Hodgkin's Disease

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CD40 is a transmembrane protein that belongs to a superfamily of proteins related to nerve growth factor receptor. CD40 is expressed on B cells and some B cell malignancies. It appears to be involved in B cell proliferation and the prevention of apoptosis in germinal center cells, which is accompanied by expression of bcl-2. Its expression is up-regulated by the EBV protein latent membrane protein-1 and cytokines interleukin-4 and interferon-γ. The expression of CD40 in 37 cases of Hodgkin's disease and 23 cases of non-Hodgkin's lymphoma (11 T cell lymphomas and 12 B cell lymphomas) was examined by paraffin section immunohistochemistry using the BB-20 monoclonal antibody. In 26 of 37 cases of Hodgkin's disease the Reed-Sternberg cells showed strong membrane or cytoplasmic expression of CD40. Only 3 of 23 non-Hodgkin's lymphomas showed any expression of CD40 and then only weakly. There was no correlation between expression of bcl-2 or latent membrane protein-1 with CD40 expression. These results show that there is probable hyperexpression of CD40 in Hodgkin's disease and suggest that dysregulation of CD40 expression may play a role in the pathogenesis of Hodgkin's disease. (Am J Pathol 1994, 144:21–26)

CD40 is a 45- to 50-kd transmembrane glycoprotein that belongs to a superfamily of cell surface proteins, some of which function as cytokine receptors.[1,2] Members of this family include nerve growth factor receptor, receptors for tumour necrosis factor, the T cell activation antigen CD27,[3,4] and the lymphocyte activation antigen CD30.[5] CD40 is found on mature B cells (except plasma cells), B cell lymphomas, some B cell acute lymphoblastic leukaemias, follicular dendritic cells, interdigitating reticular cells, and some epithelial cells and carcinomas.[2,6–8] The surface expression of CD40 is up-regulated after the activation of B lymphocytes and down-regulated on differentiation to plasma cells.

Monoclonal antibodies to CD40 mediate various effects on B cells *in vitro* including induction of short and long term proliferation,[7,9] differentiation,[8,10] homotypic and heterotypic adhesion,[11,12] and prevention of apoptosis in germinal center centrocytes.[13] These features suggest that CD40 could be a receptor with important functions in B cell development and activation. A murine ligand for CD40 has been found that mediates proliferation of both human and murine B lymphocytes in the absence of a costimulus.[14] Altered expression of CD40 antigen may thus play a role in the regulation of abnormal cell proliferation in B cell neoplasms. Although the histogenesis of Hodgkin's disease is still uncertain, with description of heterogeneous immunophenotypes and genotypes indicating that some cases are probably of T cell origin[15,16] and B cell origin, there is growing immunophenotypic and genetic evidence that in many cases Hodgkin's disease is a B cell lymphoma.[17] It is therefore possible that aberrant expression of CD40 may contribute to the pathogenesis of this lymphoma.

The purpose of this study was to investigate the expression of CD40 in Hodgkin's disease. This was determined by staining paraffin sections from cases of Hodgkin's disease with the anti-CD40 monoclonal antibody, BB20. These results were compared with the expression of CD40 seen in B or T non-Hodgkin's lymphomas and normal lymphoid tissue. The expression of CD40 was also compared with a limited panel of B and T cell markers in paraffin sections.

In view of the documented presence of EBV latent membrane protein (LMP-1)[18] in Reed-Sternberg cells and its role in the induction of CD40[19] and bcl-2[20] expression, we also investigated expression of these molecules and of the autocrine B cell growth factor CD23,[19] which is induced in B cells by EBV infection.

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Materials and Methods

Case Selection

Thirty-seven cases of Hodgkin's disease (4 lymphocyte predominant, 15 mixed cellularity, 13 nodular sclerosis, 5 lymphocyte depleted), 23 cases of non-Hodgkin's lymphoma (12 B non-Hodgkin's lymphoma; 4 follicular centroblastic-centrocytic, 4 diffuse centroblastic, 4 immunoblastic, 11 T non-Hodgkin's lymphoma; 3 T lymphoblastic, 2 T immunoblastic, 3 T large cell pleomorphic, 1 T zone lymphoma, 1 T angioimmunoblastic lymphoma, 1 mycosis fungoides), and sections of normal reactive lymph nodes and tonsils were selected from the files of the Edinburgh University Department of Pathology. All tissue samples had been routinely processed by fixation in a solution of 4% buffered formalin and embedded in paraffin wax. All the non-Hodgkin's lymphomas had been immunophenotyped previously and classified according to the modified Kiel classification.[21] The Hodgkin's lymphomas had been classified according to the Rye classification.[22]

Immunohistochemistry

Immunohistochemical staining was performed on these sections using the panel of antibodies shown in Table 1. A standard avidin-biotin complex detection method was used,[23] and endogenous peroxidase activity was blocked by incubating the sections with methanol containing 1% hydrogen peroxide for 20 minutes. Sections were incubated with the antibodies at the dilutions shown for 30 minutes at room temperature with the exception of those stained with antibody to EBV LMP, which required overnight incubation with the primary antibody at 4 C. Some of the cases included in the study had been investigated previously for the expression of CD23 and bcl-2 by staining frozen sections with bcl-2 antibody and the CD23 antibody MHM6.[24]

Table 1. *Monoclonal Antibodies used in the Study*

Antibody	Specificity	Dilution	Source
Dako M1	CD15	1/20	DAKO
L26	CD20	1/1000	DAKO
BER-H2	CD30	1/10	DAKO
BB-20	CD40	1/40	SEROTEC
MT1	CD43	1/10	Bio Nuclear Services
F8.11.13	CD45RA	1/50	R. Dalchau
UCHL1	CD45RO	1/10	P. Beverley
CS1-4	LMP	1/100	DAKO
HH-2	CD75	1/100	4th Leucocyte Workshop
BCL-2,124	BCL-2	1/10	DAKO
MHM6	CD23	1/20	DAKO

Results

Normal Lymphoid Tissues

In both tonsils and lymph nodes germinal center B cells, follicular dendritic cells, and follicular mantle zone lymphocytes showed weak membrane staining with anti-CD40 antibody BB-20 (Figure 1A). In the paracortical zones interdigitating reticular cells and occasional lymphoid blasts also showed membrane staining. There was no staining of endothelium, stroma, or tonsillar epithelium.

Hodgkin's Disease

The results of the immunostaining with the antibody panel in the cases of Hodgkin's disease are given in Table 2. In 26 of 37 cases of Hodgkin's disease there was strong membrane and/or cytoplasmic staining of the majority of Reed-Sternberg cells (Figure 1, B and C). The strength of the membrane staining was much greater than that seen in normal lymphoid tissue or in the residual follicles within tumors. CD40 expression showed no obvious relation to Hodgkin's subtype or patient's age or gender.

In 18 of 37 cases of Hodgkin's disease the Reed-Sternberg cells showed membrane expression of B cell antigens: 10 of 37 expressed CD20, 12 of 36 expressed CD75, and 6 of 37 expressed CD45RA.

Of the 26 CD40-positive cases, 13 coexpressed B cell markers CD20, CD45RA, or CD75. In eight cases only one other B cell marker was expressed, in three cases two other B cell markers were expressed, and in two cases three B cell markers were expressed in addition to CD40. Three CD40-positive cases expressed CD45RO; of these one expressed CD45RO alone, one coexpressed CD43, and the third coexpressed CD20 and CD45RA with CD43. In 11 CD40-positive cases no B or T antigens were expressed.

Immunohistochemical staining for detection of LMP was performed on 28 of the original 37 cases of Hodgkin's disease. In 12 of 28 Hodgkin's cases LMP expression was detected, confined to the Reed-Sternberg cells. As shown in Table 3 there was no correlation between expression of LMP, CD40, CD23, or bcl-2.

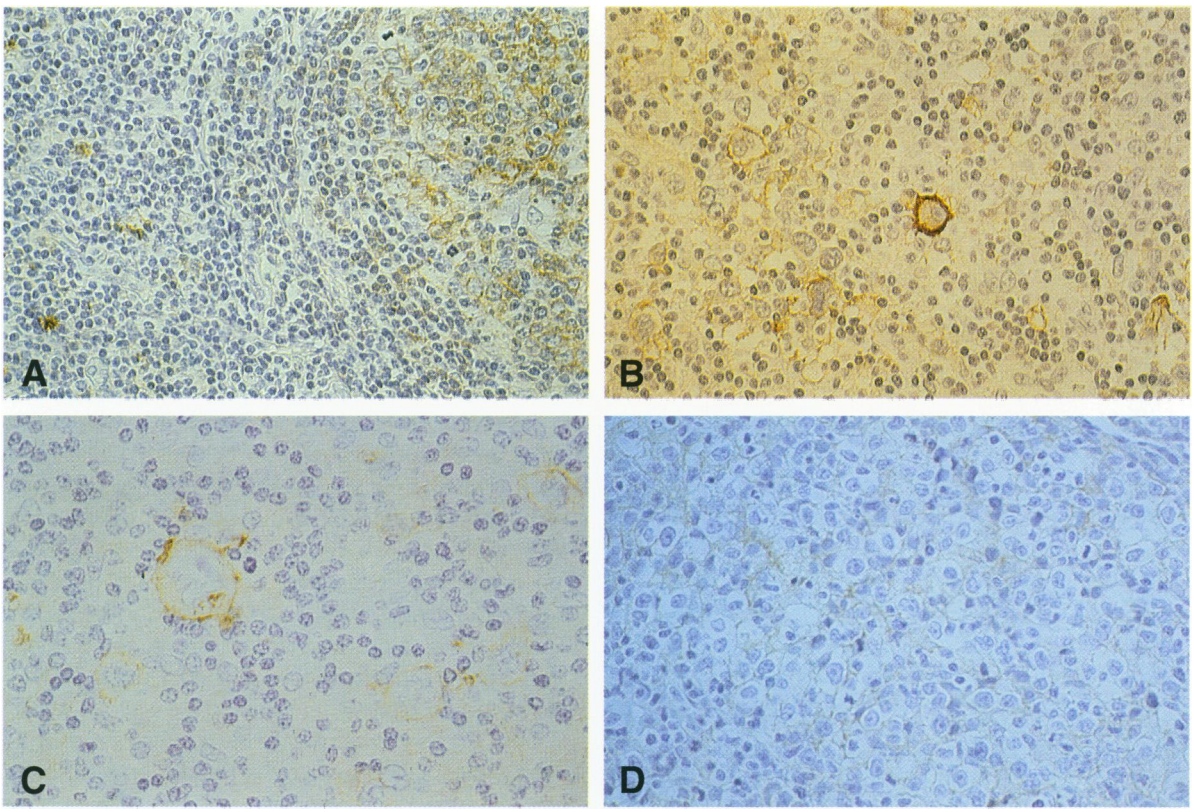

Figure 1. **A:** *Immunohistochemical staining of follicle and mantle zone cells in reactive lymph node using anti-CD40 antibody BB-20; magnification ×250.* **B:** *Immunostaining of Reed-Sternberg cells in Hodgkin's disease with anti-CD40 antibody BB-20; magnification ×250.* **C:** *Immunostaining of Reed-Sternberg cells in Hodgkin's disease with anti-CD40 antibody BB-20; magnification and ×400.* **D:** *Weak membrane staining of lymphoma cells with BB-20 antibody in a case of B immunoblastic lymphoma; magnification ×250.*

Table 2. *Pattern of Antigen Expression by Reed-Sternberg Cell in Various Subtypes of Hodgkin's Disease*

	LP	NS	MC	LD
CD40	3/4	11/13	9/15	3/5
CD20	3/4	1/13	5/15	1/5
CD75	3/4	3/13	5/15	1/4
CD45RA	3/4	2/13	0/15	1/5
CD45RO	0/4	2/13	0/15	1/5
CD15	2/2	8/10	11/14	3/5
CD30	2/2	10/10	14/14	5/5
CD43	1/4	1/13	1/15	0/5
Total cases	4	13	15	5

LP, lymphocyte predominant; NS, nodular sclerosis; MC, mixed cellularity; LD, lymphocyte depleted.

Non-Hodgkin's Lymphoma

Only 3 of 23 non-Hodgkin's lymphomas expressed CD40 in paraffin sections including one B immunoblastic lymphoma (Figure 1D), one follicular lymphoma, and a T lymphoblastic lymphoma. All three showed only weak staining with BB-20 in comparison to that observed in Reed-Sternberg cells. There was no LMP expression in any of the non-Hodgkin's lymphomas including these three cases.

Discussion

Despite extensive study the histogenesis of Reed-Sternberg cells remains controversial. It is likely that Hodgkin's disease is a heterogeneous disorder and includes cases of both T and B cell origin.[15–17] There is, however, growing immunophenotypic and genetic evidence that in many cases Hodgkin's disease is a B cell neoplasm and for this reason we investigated the expression of CD40 on Reed-Sternberg cells because this molecule is known to be important in B lymphocyte growth regulation. CD40 is a member of the nerve growth factor receptor family, which also includes CD30, a lymphocyte activation antigen that is commonly expressed by Reed-Sternberg cells.[5]

The expression of CD40 by Reed-Sternberg cells has been previously reported,[25] but was interpreted as demonstrating a follicular dendritic cell origin. Using an antibody panel that included B cell markers in addition to CD40, we have demonstrated strong expression of CD40 in most cases (26 of 37) of Hodgkin's disease that we studied. Furthermore, we were able to demonstrate coexpression of CD40

Table 3. *Expression of CD40, bcl-2, CD23, and LMP-1 in Reed-Sternberg Cells*

Subtype	CD40	LMP-1	bcl-2	CD23
LD	+	+	ND	ND
LD	+	+	–	–
LD	–	–	ND	ND
LP	+	+	ND	ND
LP	+	–	ND	ND
LP	+	–	–	–
LP	–	–	ND	ND
MC	+	–	–	–
MC	+	+	ND	+
MC	–	–	–	–
MC	+	–	+	+
MC	+	–	+	–
MC	+	–	+	+
MC	+	+	+	–
MC	+	+	–	–
MC	–	+	ND	ND
MC	–	+	ND	ND
MC	–	+	ND	ND
MC	–	+	ND	ND
MC	+	–	ND	ND
NS	+	–	ND	ND
NS	+	+	+	+
NS	+	–	ND	ND
NS	+	–	–	–
NS	+	+	–	–
NS	+	–	ND	ND
NS	–	–	ND	ND
NS	+	–	ND	ND

ND, not determined; +, positive staining; –, negative.

and B cell antigens confirming that these tumors were of B cell origin. This expression showed no obvious relation to Hodgkin's subtype, stage, or patient's sex or age. Of the 26 CD40-positive cases 13 coexpressed one or more of the B cell markers examined. These results are consistent with a B cell origin in most cases. Three cases of Hodgkin's disease did express T cell markers. In these cases the Reed-Sternberg cells also expressed CD40 and one of these also coexpressed B cell markers CD20 and CD45RA.

The reasons for the high levels of CD40 expression on Reed-Sternberg cells remain unclear, although there are several possible explanations. CD40 expression may not be properly down-regulated after combination with its ligand, possibly because the molecule is altered in the malignant cell. Alternatively, CD40 expression is known to be up-regulated by the cytokines interleukin (IL) 4 and interferon-γ[2,26] both of which have been reported in Hodgkin's tumors,[27] and by EBV LMP,[19] which is expressed by Reed-Sternberg cells in many cases of Hodgkin's disease.[18] Any one or a combination of these events may account for the strong surface staining observed in Reed-Sternberg cells.

Whatever the reasons for the up-regulated expression of CD40 it may confer a growth advantage on the cells expressing it, assuming that it retains the same properties that it has in normal B cells. In support of this it is known that some malignant B cells retain the capacity to respond to anti-CD40 antibodies by proliferation[8] and that CD40 is highly expressed by rapidly growing B cell lines, eg, Burkitt's lymphoma cell lines and EBV-transformed lymphoblastoid cultures as opposed to the relatively moderate expression in most freshly isolated B cells of normal or malignant origin.[6] Elevated expression of CD40 may promote proliferation by increasing the number of binding sites available for its physiological ligand or play a role in the prevention of apoptosis.

The findings of Armitage, et al[14] of a murine ligand for CD40 expressed on activated T cells that also acts on human B cells is provocative. This membrane-bound ligand is capable of delivering a directly mitogenic signal to resting B cells without the need of a costimulus. Spriggs, et al have recently identified a human CD40 ligand restricted to T cells that also provides a directly mitogenic signal to B cells in the absence of a costimulus.[28] This raises the possibility that the reactive infiltrate of activated T cells in Hodgkin's disease may be providing a proliferative signal to the Reed-Sternberg cells and the enhanced heterotypic adhesion induced by signaling through CD40 would facilitate this interaction.[11,12] This may explain the difficulty of growing these tumors *ex vivo* and the more indolent progression of lymphocyte predominant Hodgkin's disease in which the background infiltrate is predominantly B cell.

It is also possible that a soluble CD40 ligand may be secreted by T cells or even B cells. Delivery of a proliferative signal by a soluble ligand usually requires a costimulus but appropriate costimuli could be found in Hodgkin's disease including IL-4[9] and IL-10. The latter is a T helper 2 cell product that is highly homologous to BCRF-1 (viral IL-10), an open reading frame in the EBV genome. Human and viral IL-10 have been shown to stimulate DNA replication of B lymphocytes activated via their CD40 antigen.[29]

The prevention of apoptosis via the CD40 pathway or via the CD23/IL-1α pathway is accompanied by the induction of bcl-2 protein expression.[30] LMP-1 is also known to up-regulate bcl-2 expression,[20] however, we could detect no correlation between the expression of bcl-2 and CD40, CD23, or LMP-1. This may indicate signal uncoupling or the use of an alternative pathway to prevent apoptosis.

Only three of the non-Hodgkin's lymphomas expressed CD40. This is in contrast to reported results described using flow cytometry where CD40 ex-

pression is described in most B cell and occasionally in T cell lymphomas.[31] This may reflect loss of antigen during processing. The fact that strong expression of CD40 could be seen in most cases of Hodgkin's disease after identical processing suggests that Reed-Sternberg cells show a higher level of expression of CD40 than the cells of non-Hodgkin's lymphomas. The possibility remains that this observed difference represents expression of different isoforms of CD40 by different cell populations. Because it is known that CD40 is more highly expressed in rapidly proliferating neoplasms than in normal B cells or slowly proliferating neoplasms, CD40 expression may provide an additional index of proliferation or prognosis especially in non-Hodgkin's lymphomas.

Thus, it seems that in Hodgkin's disease there are several reasons for the up-regulation of CD40 and abundant availability of putative ligands combined with an appropriate environment resulting in proliferation of the putative target B cell. The fact that the CD40 antigen is not lost unlike many other B cell surface antigens related to proliferation may suggest that its continued expression on the cell surface is important for tumor survival at least in some cases of Hodgkin's disease.

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