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Abundant expression of CD40 and CD40-ligand (CD154) in paediatric Langerhans cell histiocytosis lesions

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Abstract

The pathogenesis of Langerhans cell histiocytosis (LCH) is obscure, partly because the events leading to activation of Langerhans-like lesional cells (LCH cells) and associated T cells, and the excessive cytokine production by these cells are unknown. The interaction between CD40 on antigen-presenting cells (APC) like Langerhans cells and CD40 ligand (CD40L) (CD154) expressed by activated CD4+ T cells, is essential for the activation of both the APC and the T cells and results in upregulation of APC functions and initiation of immunoreactivity. The effects of CD40–CD40L interaction include increased expression of co-stimulatory and adhesion molecules, proliferation, and production of pro-inflammatory cytokines and proteolytic enzymes, all features of LCH. Using immunohistochemistry, we analysed the *in situ* presence of the co-stimulatory molecules CD40 and CD40L in 15 fresh frozen biopsies of LCH lesions in children. The cells producing these molecules were identified by double staining for CD1a on LCH cells and CD3 on T cells. Prominent expression of CD40 by LCH cells and CD40L by T cells was found in all 15 specimens regardless of the source of specimen or characteristics of the patient. The findings of high expression of CD40 and CD40L in all specimens imply a key role for the CD40–CD40L adhesion pathway in the pathogenesis of LCH. Since this interaction is an accessible and realistic target for immunotherapy, these findings prompt speculation on the use of blocking antibodies to CD40 or to CD40L in the treatment of LCH. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Langerhans cell histiocytosis; Co-stimulatory molecules

1. Introduction

Langerhans cell histiocytosis (LCH) is a rare disorder with a broad clinical spectrum that ranges from mono-osteolytic lesions that are generally cured by curettage, to a disseminated disorder which occurs in young children, and that has substantial morbidity and mortality [1]. Histologically, the lesions of LCH are polymorphous, usually vary little from site to site and from patient to patient, and the predominant cells are CD1a+ (clusters of differentiation) histiocytes, some of which bear Birbeck granules, and T cells [2]. In characteristic LCH lesions of lymph nodes, T cells account for approximately 20% of lesional cells, LCH cells for 40%, macrophages for 35%, eosinophils 5% and B cells 1%. Typically 15% of the histiocytes and 2% of the T cells are cycling [3]. Comparable data on bone lesions are not available, but there is likely to be little difference. The stereotypical LCH lesion features a monoclonal population of LCH cells, akin to the Langerhans cell [4]. Although the LCH cell seems to be a key player in LCH lesions, CD3+ T cells are also constant and often prominent.

Recently, we showed that lesions of LCH are also rich in various cytokines that are produced by these two elements [5]. Antigenic peptide-specific interactions between T cells and antigen presenting cells (APC), with

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which the LCH cell shares many features, are to a large extent guided by membrane-bound co-stimulatory receptor–co-receptor pairs, such as CD40–CD40 ligand (CD40L) and CD28/cytotoxic T lymphocyte associated molecule 4 (CTLA4)–CD80/CD86 (B7-1/B7-2). Adhesion molecules and cytokines/chemokines provide additional signals. Ligation of CD40 expressed by HLA class II positive APC, is an early and pivotal signal for the upregulation of APC and the induction of diverse effector functions [6,7]. In addition, ligation of CD40L expressed by activated CD4+ T cells is crucial to T cell priming [8] and T cell cytokine production [9]. Thus, the CD40–CD40L adhesion pathway is an integral part of bi-directional T cell/APC communication, stimulating functional activities by both parties. Importantly CD40–CD40L interactions can also lead to cellular activation in the absence of HLA-peptide/T cell receptor ligation, since recombinant CD40L alone is sufficient to activate CD40+ APC *in vitro*. This implies that CD40–CD40L interactions can be pathophysiologically relevant even in the absence of (auto)-antigen presentation. Immunotherapy, targeting these molecules, need not rely on the positive identification of (auto)-antigens.

To further elucidate the cellular interactions in LCH lesions, we assessed the expression of CD40 on LCH cells and CD40L on T cells in LCH lesions from 15 paediatric patients. During the course of this study, it was reported that in adult pulmonary LCH the expression of CD80 and CD86 molecules was upregulated by CD40–CD40L interaction [10]. In these adult cases of pulmonary LCH, LCH cells expressed CD40 and approximately one-third of the T cells in the lesion expressed CD40L [10]. Although in paediatric patients with LCH the expression of CD80/CD86 molecules have been detected [11], there is no information on the CD40–CD40L pathway. Furthermore, there are no data on this interaction in organs other than lung that are more often involved in LCH in children.

2. Patients and methods

The immunohistochemical methods used have been described in detail previously [12–14]. Fifteen LCH biopsy specimens were snap-frozen and kept at −80°C until frozen sections were cut from each. Briefly, frozen sections were cut 8 microns apart in an environment of −20°C. A series of seven sections from each specimen together with sections of appropriate control tissue were placed on individual glass slides, and were kept overnight at room temperature in a loosely closed container with humidified atmosphere. After undergoing air-drying for 1 h, sections were fixed in acetone containing 0.01% (v/v) hydrogen peroxide to block endogenous peroxidase activity. Sections were then air-dried for 10 min and incubated overnight with optimally diluted primary antibodies (5–0.5 μ/ml) at 4°C in the dark. Sections were washed three times in phosphate buffered saline (PBS) and incubated with secondary and tertiary reagents for 1 h at room temperature, with washing in PBS between all incubation steps. HRP (peroxidase) activity was revealed using 3-amino-9-ethylcarbazole (AEC) producing a translucent red precipitate. Alkaline phosphatase activity was revealed using naphthol-AS-MX phosphate and Fast Blue BB Base, producing a dark blue precipitate.

For combined immuno- and histochemistry, histochemical revelation of acid phosphatase activity, a lysosomal enzyme of monocytic cells, was performed by incubating fixed sections with naphthol-AS-BI phosphate and pararosanilin in sodium acetate/barbital sodium buffer for 30 min at 37°C, resulting in a bright red intracellular staining. This was done one step prior to revelation of alkaline phosphatase activity, the enzyme linked to the final antibody reagent, which produces a dark blue precipitate (detection of membrane-expressed CD40).

CD3 was detected by Dako-EPOS (Dako, Copenhagen, Denmark), a rabbit polyclonal antibody coupled to an inert polymer backbone and HRP (horseradish peroxidase). CD1a was detected by antibody 1/34 (Dako). CD40L-expressing cells were detected immunohistochemically by using a biotinylated CD40-Ig fusion protein (a gift of R.J. Noelle, Dartmouth College, Lebanon, NH, USA) and avidin-HRP (Dako) [15]. CD40 was demonstrated using a mouse anti-human CD40 antibody (5D12; a gift from M. de Boer, Tanox Pharma BV, Amsterdam, The Netherlands) followed by horse anti-mouse Ig biotin (Vector, Peterborough, UK).

The specificity of antibodies was determined as noted earlier [13], but isotype-matched control antibodies were always included to assess non-specific background staining (e.g. by binding to Fc receptors). When stains could not be reproduced because of lack of tissue or lack of tissue integrity and/or lack of clearly discernible cytology, they were shown as (+) in Table 1. Negative controls uniformly showed no staining. Selected central nervous system tissues from multiple sclerosis patients ($n=4$) in which CD40 and CD40L were detected in previous studies were used as positive tissue controls [16]. Identity of the cells was determined by double staining for CD1a for LCH cells and CD3 for T cells as described in [5]. All stains were done in duplicate on separate slides on the same day, and duplicate stains were performed on different days. Sections were briefly counterstained with haematoxylin and embedded with glycerol-gelatine. As in earlier studies [13], cells were scored as positive only when the cytoplasm was brightly coloured and the nucleus was unstained. Two observers scored all slides independently and reconciled occasional differences in scoring by studying the slide(s) together. Slides were scored for estimates of the number of positively staining lesional cells. Double staining for

cell type and co-stimulatory molecule was performed in at least seven sections for each profile. Eosinophils were identified on the basis of morphology and CD1a negative cells with morphological features of histiocytes, were called macrophages.

3. Results

3.1. *Tissue specimens*

A definitive diagnosis of LCH and the representative nature of 15 specimens from the 15 patients were confirmed by one of the investigators. Twelve (80%) of the specimens were from bone (mono-ostotic LCH) and 3 (20%) were from excisional lymph node biopsies from patients with disseminated LCH. All specimens were from initial diagnostic biopsies, the duration of symptoms and signs being less than a month in all cases. Selected characteristics of patients and biopsies are listed in Table 1.

3.2. *High expression of CD40 and of CD40L in LCH lesions*

Both CD40 and CD40L were expressed in all LCH specimens evaluated (Table 1). Two specimens had very large numbers of CD40-expressing cells, as shown in Fig. 1(a), but these biopsies did not have large numbers of CD40L-expressing cells. In contrast, three biopsies showed large numbers of CD40L-expressing cells (see Fig. 1b), but CD40 expression was not high in these specimens. High expression levels of either CD40 or its ligand did not seem to correlate with specimen source or patient characteristics.

Table 1
Immunohistochemical analysis of cell determinant 40 (CD40) and CD40L (ligand) in Langerhans cell histiocytosis (LCH) biopsies[a]

Patient	LCH lesion	Age in years/Sex	CD40	CD40L
9408P025	Mono-ostotic: fibula	1/M	+	+
S943463	Mono-ostotic: skull	5/F	+	+
9101115	Mono-ostotic: skull	5/F	++	+
94111100	Mono-ostotic: skull	5/M	+	+
9411139	Mono-ostotic: pelvis	8/M	+	(+)
9408P022	Mono-ostotic: mastoid	9/M	(+)	+
S926704	Mono-ostotic: skull	10/F	+	++
9101118	Mono-ostotic: femur	10/M	++	+
9407159	Mono-ostotic: skull	11/M	+	+
9411142	Mono-ostotic: skull	11/M	+	(+)
9209EE2	Mono-ostotic: skull	12/M	+	(+)
CS21789	Mono-ostotic: rib	12/M	+	(+)
S93292	Disseminated: lymph node	1/M	+	++
9307142	Disseminated: lymph node	2/M	+	(+)
R94101	Disseminated: lymph node	2/F	+	++

[a] Staining was performed in duplicate on separate glass slides and twice on different days (minimum of four data points) in comparison to known positive controls (selected central nervous system tissues from multiple sclerosis patients). ++, high number of cells: +, moderate number of cells: –, few to no cells expressing co-stimulatory molecules: (+), insufficient tissue remaining for repetition of the experiment.

3.3. *Co-localisation of CD40 and CD1a on LCH cells and of CD40L and CD3 on T cells in LCH lesions*

Double labelling experiments revealed that CD40L was exclusively expressed by CD3+ T cells (see Fig. 2a). In contrast, CD40-expression was restricted to CD1a+ LCH cells (data not shown). This is in agreement with the APC-like morphology and phenotype (e.g. HLA class II) of LCH cells and the fact that activated CD4+ T cells are the main source of CD40L expression in physiological immune responses [6–8]. The macrophages and eosinophils present in the lesions did not stain for either CD40 or its ligand, and there were insufficient numbers of multinucleated giant cells to judge their expression of CD40 or CD40L by these

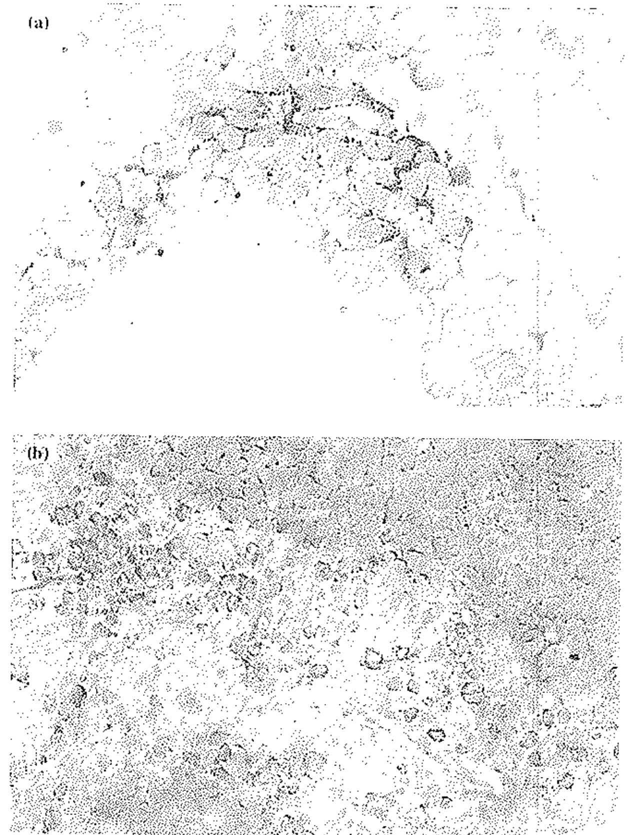

Fig. 1. *In vivo* evidence for high expression of CD40 and CD40L in Langerhans cell histiocytosis (LCH) lesions. (a) CD40 bearing LCH cells in red as demonstrated followed by horse anti-mouse Ig biotin, avidin-HRP and AEC staining (magnification ×100). (b) CD40–ligand bearing T cells in red as demonstrated with biotinylated CD40-Ig, followed by avidin–HRP and AEC staining (magnification ×50).

elements (data not shown). Cells expressing CD40 and cells expressing CD40L were found in close juxtaposition after double staining (see Fig. 2b), suggesting ongoing cellular interactions.

4. Discussion

This work demonstrates that LCH lesions in bone and lymph nodes feature a milieu in which large numbers of characteristic CD1a+ histiocytes and CD3+ T-cells express high levels of CD40 and CD40L, respectively. There is currently no reason to think that LCH in the skin and/or in visceral organs exhibits another pattern. Previous studies have shown that the lesion of LCH is also rich in various cytokines that are produced by these two elements [5]. These findings are important for two reasons. Firstly CD40 ligation may explain both the intense proliferation of LCH cells and the cytokine storm that is so characteristic of the LCH lesion. Secondly, the CD40–CD40L interaction is an accessible and realistic target for immunotherapy of LCH.

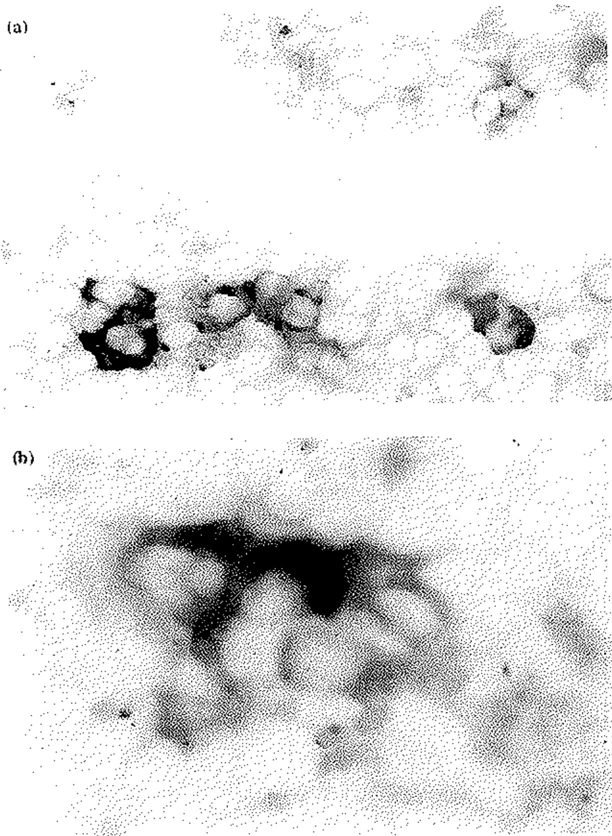

Fig. 2. *In vivo* evidence for involvement of CD40–CD40L interactions in Langerhans cell histiocytosis (LCH) lesions. (a) CD40L is stained in blue and CD3 in red. Hence, the violet colour indicates that CD40L-expressing cells co-express CD3 implying that they are T cells (magnification ×100). (b) CD40L is stained in blue and CD40 in red. Cells expressing CD40 and CD40L are directly juxtaposed.

CD40 ligation on 'normal' Langerhans cells leads to upregulation of the co-stimulatory molecules CD80 and CD86, the adhesion molecules CD54 (ICAM-1) and CD58 leucocyte function (LFA-3), interleukin (IL)-2R and major histocompatibility complex (MHC) Class II (through interferon (IFN)-γ) [16–18]. In LCH, however, few studies addressing the co-stimulating pathways have been undertaken. De Graaf and colleagues stated that LCH cells express a pattern of activated Langerhans cells, by showing strong expression of CD54/ICAM-1, CD58, CD49d/VLAα4 in combination with high expression of the β1 and β2 chains of the integrins [17]. Emile and his co-workers came to the same conclusion by showing expression of LFA-1, CD24, CD86 and CD80 markers on the LCH cells [11]. The strong positivity for CD80/CD86 was recently confirmed in adult pulmonary LCH [10]. The CD40–CD40L adhesion pathway on LCH cells could also increase the expression of CD80 and CD86 molecules, and improve their lympho-stimulatory activity. In order to correlate expression and function further *in situ/in vitro* analysis should take into account several sources of variation, such as lesions in different organs, the 'age' and activity of the lesion and the different components of the lesion.

The CD40 ligation of LCH cells and the large number of lesional CD40L+ T cells may explain the seemingly erratically and uncontrolled production of various cytokines in LCH lesions. The ligation of CD40L on activated T cells is required for T cell priming [8]. Likewise, the production of Th1 and Th2 cytokines, such as IL-2, IL4, IL-5 and IFN-β, is enhanced by CD40L ligation. These same cytokines have been shown to be produced by large numbers of cells in LCH lesions. Thus, CD40 ligation may mediate signalling that ultimately causes activation of the antigen-presenting LCH cell and the T cells in LCH lesions. Since T cells in LCH have a small cycling index [4] it is likely that this activation is manifested as cytokine production, rather than proliferation. CD40 ligation also upregulates IL-2R on Langerhans cells, which in conjunction with the increased IL-2 production in LCH lesions, may partially account for the cytokine cascade in this disorder.

Under physiological conditions, CD40 ligation influences dendritic cell maturation, rescues Langerhans cells from apoptosis and plays a role in T cell activation [19]. The upregulation of the co-stimulatory and adhesion molecules like CD80, CD86, CD54, CD58, IL-2R and MHC Class II through CD40 ligation on Langerhans cells, promote increased antigen presentation, proliferative responses and INF-γ production by T cells [20]. IFN-γ, in turn, enhances IL-1α production by Langerhans cells [21]. The large amounts of IFN-γ produced by LCH cells and T cells in the lesions of LCH might stimulate the production of IL-1α, which in turn activates osteoclasts leading to bone resorption in LCH [5,22]. This process is enhanced by tumour necrosis

factor (TNF)-α derived from T cells in these lesions. Furthermore, IL-1α has a synergistic effect on CD40L and TNF-α in the rescue of Langerhans cells from apoptosis that is supported by increased production of granulocyte macrophage-colony stimulating factor (GM-CSF) and IL-3 [19,23–25]. The lesional accumulation of LCH cells may be explained on this basis. The cytokines in LCH lesions, including IL-3, GM-CSF, TNF-α are chemo-attractants for eosinophils, neutrophils, macrophages and CD34+ Langerhans cell progenitors, and cause transformation of the latter into Langerhans cells [24,26]. Additionally, lesional GM-CSF and TNF-α of T cell origin likely promote CD40 expression by LCH cells. The triggering of CD40L on T cells can support regulated LCH cell and T cell expansion, as well as cytokine production. IL-10, in contrast, counteracts the effects of CD40L and TNF-α. LCH lesions contain abundant TNF-α, but little IL-10 in most specimens [5].

Treatment of patients with LCH depends on the extent of disease. Based on the results of the study presented here, therapeutic strategies using intact antibodies, fusion protein constructs or small molecule inhibitors to block the CD40–CD40L interaction might be considered [27,28].

Although the concept of activation of LCH cells, as well as the role of CD40 ligation in dendritic cells is known, the exact meaning of these features in LCH remains to be determined in more extensive studies. Ongoing work on the CD40–CD40L interaction and its role in LCH should increase our understanding of this disease and determine if this pathway is unique to LCH.

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