Distribution of OX2 Antigen and OX2 Receptor within Retina

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Purpose. OX2 is a member of the immunoglobulin superfamily expressed on a broad range of tissues including neurons of the central and peripheral nervous systems, thymocytes, and endothelium. The recently identified OX2 receptor (OX2R) is restricted to the surfaces of myeloid lineage cells, including microglia. Functional data have implicated the OX2–OX2R interaction as a myeloid downregulatory signal. The purpose of this study was to determine the distribution and extent of expression of OX2 and its receptor within the retina, a tissue developed to restrain immunemediated inflammatory damage.

Methods. OX2 and OX2R monoclonal antibodies (mAbs) were used to determine OX2 and OX2R protein expression, respectively, by flow cytometry of isolated myeloid-derived cells from normal and inflamed rat retina and by immunohistochemistry of serial sections of rat retina. For comparison, distribution of OX2 was documented using species-specific monoclonal antibodies in mouse and human retina. No OX2R mAbs are available for mouse or human detection.

Results. OX2 was expressed on retinal vascular endothelium and glial fibrillary acidic protein (GFAP)-negative neurons in retina and optic nerve and on a subpopulation of CD45+ perivascular and juxtavascular cells. Within normal retina, OX2R was not detected on myeloid-derived cells. During experimental autoimmune uveoretinitis (EAU), expression of OX2 and OX2R was noted on infiltrating leukocytes.

Conclusions. Taking these results of the distribution of OX2 in normal and OX2R in inflamed retina with other functional data of OX2–OX2R interaction, it is suggested that the OX2–OX2R interaction has the potential to contribute to a novel pathway that suppresses and limits immunologic inflammatory damage within the retina. (Invest Ophthalmol Vis Sci. 2001;42:170–176)

A member of the immunoglobulin superfamily (IgSF), OX2 is a 41- to 47-kDa cell surface glycoprotein containing two IgSF domains in a typical V-/C2 set arrangement. In rats, OX2 has a wide distribution, and expression has been reported on neurons, activated T-cells, B-cells, follicular dendritic cells, and endothelium.1–3 OX2 is structurally similar and genetically linked to costimulatory molecules B7-1 and B7-2,4 and recent data have indicated both stimulatory5 and tolerogenic6–8 roles for OX2 in antigen presentation. However, given the wide distribution of OX2, particularly on neurons,9 a primary role for OX2 in antigen presentation has yet to be confirmed. A multivalent OX2-binding reagent detected a receptor for OX2 on resident peritoneal macrophages of both rats and mice.10 More recently, molecular identification of the receptor for OX2 (OX2R) showed that in contrast to OX2 itself, the OX2R was restricted to cells of the myeloid lineage (G.J. Wright, manuscript submitted, 2000). In support of these findings, the phenotype of an OX2-deficient mouse showed defects in myeloid cellular biology within tissues that express OX2. These defects included elevated numbers of macrophages in the spleen and an increase in number and activation state of brain microglia (MG; J.D. Sedgwick, manuscript submitted, 2000). This phenotype suggests that tissues expressing OX2 are able to deliver a negative regulatory signal, through the OX2R and thereby regulate macrophage biology. The loss of some microbial regulatory mechanism in the knockout mouse suggests that the OX2–OX2R interaction may be, at least in part, responsible for the immune privileged status of the central nervous system by limiting the activity of myeloid cells.

Traditionally, the status of immune privilege of the retina is afforded because of a paucity of antigen-presenting cells (APCs) and the blood-retinal barrier, although immunemediated tissue damage is readily elicited in experimental models (e.g., experimental autoimmune uveoretinitis [EAU]) and is not uncommon clinically. Previous data have led to the postulation that immune privilege of the cornea and retina from immune-targeted damage may in part be mediated through constitutive expression of Fas ligand on, for example, corneal endothelium and retinal pigment epithelium.11,12 Local mechanisms whereby immune-mediated retinal damage is regulated, particularly control of infiltrating macrophages, remain largely unresolved.13 Additionally, although immune privileged, the retina contains two populations of myeloid-derived cells.14 Due to their cell surface phenotype, MG are implicated as putative APCs.15 To date, similar to central nervous system [CNS] MG, there are no functional data within the retina to support such a functional implication.16 The documentation of neuronal OX2 expression and OX2R expression restricted to myeloid lineage proffers an investigation into the role that this interaction may play within the retina. Further understanding of such interactions will assist in our understanding of retinal MG biology, the control of macrophage activity during development, and inflammatory and degenerative disorders. The localization of both OX2 and its receptor were therefore determined in both healthy and inflamed (EAU-induced) tissue.

Methods

Monoclonal Antibodies

Species-specific monoclonal antibodies (mAbs) were used to detect expression of OX2 or OX2 in human, mouse, and rat retina by immunohistochemistry and flow cytometry. Unconjugated mouse anti-human and mouse anti-rat OX2 mAbs were obtained from Tetralink (Ontario, Canada). Mouse anti-rat OX2 and OX102 (anti-rat OX2R; expressed only on rat macrophages and blocks OX2 binding) were generated in the former Medical Research Council (MRC) Cellular Immunology Unit (Oxford UK). For both dual immunohistochemistry and three-color flow cytometric analysis of rat retina, mouse mAb against major histocompatibility (MHC) class II (OX6), CD45 (OX1), CD11b/c (OX42), and R73 (rat T-cell receptor) were obtained from

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Supported by the Iris Fund.

Submitted for publication March 14, 2000; revised June 16, July 31, and August 23, 2000; accepted September 6, 2000.

Commercial relationships policy: N.

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Serotec (Oxford, UK). mAb for glial fibrillary acidic protein (GFAP) was obtained from Sigma-Aldrich (Poole, UK). For analysis of human and mouse retina mouse or rat mAb against CD45, CD11b, CD31 (anti-PCAM) and MHC class II (HLA-DR) were obtained from PharMingen (San Diego, CA). mAbs were used either purified or directly conjugated to fluorescein isothiocyanate (FITC), R-phycocerythrin (PE), or biotin, as required. Biotin antibodies were labeled with streptavidin-allophycocyanin (SA-APC, supplied by PharMingen) for subsequent flow cytometric detection.

Retinal Tissue

Human donor tissue was received from the Amsterdam Eye Bank with consent of the donors and in accordance with the tenets of the Declaration of Helsinki after the removal of corneas for transplantation. Retina from adult Lewis rats (150–200 g, 6 weeks of age; Harlan and Olac, Crawley Down, UK) were obtained from both healthy animals and animals with EAU. EAU was induced by a 0.1-mL intradural injection of 6 mg/ml of soluble bovine retinal extract (RE) vol/vol in complete Freund’s adjuvant (CFA; enriched with 0.6 mg H37RA Mycobacterium tuberculosis; Difco; Detroit, MI), as previously described.17 All experiments complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and followed United Kingdom Home Office guidelines for animal welfare. Mouse retina from healthy animals was obtained from both B10.RIII and C57/BL6 strains, inbred in the animal facility of the medical school, University of Aberdeen (UK). Retina from mice with EAU were also analyzed. EAU in B10RIII strain was induced by a 0.1-mL intradural injection of interphotoreceptor binding protein (IRBP) with CFA (vol/vol) and additionally 1 μg in 0.25 ml of Bordetella pertussis toxin (Speywood, Clwyd, UK) intraperitoneally, as described.18 EAU in the C57/BL6 strain was induced by 300 μg of synthetic peptide 1-20 of IRBP (kindly provided by Arthur Moir, University of Sheffield, UK; sequence GPTFLQPSVLDMAKVL) with CFA (vol/vol) and addition of 1 μg pertussis toxin intraperitoneally.19

Immunohistochemistry

Human eyes were cut into small sections consisting of sclera, choroid, and retina, and whole eyes of rodents were fresh frozen in optimal cutting temperature compound (OCT). Serial sections were cut, air dried, and fixed in 100% acetone. After rehydration in Tris-buffered saline (TBS), sections were blocked with TBS-1% bovine serum albumin (BSA). Staining was performed with primary mAb (OX2 for human and rat or OX102 for rat only) as a spent tissue culture supernatant in 10% fetal calf serum (FCS) and detected with a secondary antibody, a biotin-labeled horse anti-mouse IgG (Vectastain; Vector Laboratories, CA) visualized using streptavidin and biotinylated horseradish peroxidase complex (SABC) and diaminobenzidine tetrahydrochloride (DAB).

After further washing in TBS-1%BSA and blocking in normal horse serum and avidin-biotin block (Vectastain; Vector), the second mAb was used at previously optimized dilutions for each species (CD45, MHC class II, OX42, GFAP, and CD11b). Secondary biotin-labeled antibody was visualized using SABC and alkaline phosphatase antialkaline phosphatase (APAAP) substrate. Levamisole (Sigma) was added to the AP substrate to block endogenous alkaline phosphatase activity. Negative controls were isotype matched, and sections were lightly counterstained in dilute hematoxylin. Single and dual immunofluorescence staining was also performed. Sections were prepared according to these procedures for color staining, except that anti-mouse FITC was added after incubation with unconjugated mAb. For double staining streptavidin Texas red was applied after secondary biotinylated antibody instead of the color substrates. Single stains were counterstained with propidium iodide.

Flow Cytometric Analysis

Eyes were dissected by removal of the iris, lens, and vitreous. The retina was then microscopically dissected and mechanically disrupted by passing through a metal sieve (250 μm). After washing in PBS-1% BSA cells were enzyme treated with collagenase (5 U/retina) and DNase (15 U/retina; Boehringer Mannheim, Mannheim, Germany) for 30 minutes at 37°C. After further washes, resident and infiltrating leukocytes were purified over a Percoll density gradient (PharMacia, Uppsala, Sweden), as previously described.20 For three-color flow cytometric staining, species-specific mAbs were added sequentially at optimized concentrations. After washing in fluorescence activated (FACS) buffer (1% BSA, phosphate-buffered saline [PBS], 10 mM Na2PO4) and primary incubation with unconjugated mAb and FITC-conjugated Fab(ab’2) anti-mouse immunoglobulin (Sigma), cells were blocked with 10% normal mouse serum (NMS) and normal rat serum (NRS). Further antigen expression was detected with biotin-conjugated mAb and APC-conjugated mAb. Biotin-conjugated mAbs were detected after further incubation with streptavidin-PE. A total of 10,000 events were acquired (FACScalibur; Becton Dickinson, Mountain View, CA) and analyzed using acquisition and analysis software (CellQuest; Becton Dickinson). Appropriate leukocyte gate and instrument variables were set accordingly. Isotype controls were included, and analysis of fluorescence was performed after further backgating to exclude dead cells and aggregates. Extent of fluorescence intensity of isolated populations was recorded as mean fluorescence intensity (MFI).

RESULTS

Distribution of OX2 Expression within Normal Retina

In normal rat and human retina, widespread OX2 expression was seen on both myeloid- and non–myeloid-derived cells, including GFAP-negative neurons and inner retinal vessel endothelium (Fig. 1 and Fig. 2). OX2 expression was also detected within GFAP-negative neurons within optic nerve (data not shown). Dual immunohistochemistry confirmed that in normal retina a population of both perivascular and juxtavascular CD45+ cells were OX2 positive (Figs. 2D, 2E). Moreover, in humans, previous reports that CD45+ myeloid-derived cells within retina were also MHC class II–positive were confirmed, the presence of OX2+ MHC class II+ cells was demonstrated (Figs. 2A, 2B, 2C). Dual staining, by both immunohistochemistry and immunofluorescence (Figs. 2F, 2G, 2H) confirmed that the majority of OX2+ cells were not GFAP+ glial cells. Because of the intensity of GFAP staining as a result of nonspecific activation of macroglia in human retina obtained after death, most OX2 expression was masked, as confirmed by flow cytometric analysis (Figs. 3 and 4). OX2 was expressed on retinal vessel endothelium (Figs. 2I, 2J). Not all MHC class II+ or CD45+ cells (Figs. 2A through 2E) expressed OX2, and therefore most constitutive OX2 expression within retina can be accounted for by endothelial and neuronal cells.

OX2 expression increased during immune-mediated inflammation, as shown in Figure 1E from a retina of a patient with sympathetic ophthalmia. Using flow cytometric analysis and confirmed in three independent experiments, with the advantage of sampling the whole tissue as a single-cell suspension, myeloid from nonmyeloid cells could be identified easily. Furthermore, resting retinal MG characterizedly expressed the cell surface phenotype CD45lowCD11b/c+.17 Therefore three-color flow cytometric analysis confirmed OX2 expression on CD45+ cells in both rat and human. In rat, however, the majority of CD45lowCD11b/c+ MG (Figs. 3E, 3F) were OX2 negative or showed low expression. In both rat and human retina, OX2 expression was between 5 and 10 times greater on myeloid-derived cells than on non–myeloid-derived (CD45−) cells (Figs. 3C through 3F). CD45-negative cells represent neuronal elements and cell bodies, because the majority of endothelial cells are removed during purification.17 In the mouse, immunohistochemical analysis with the available mAb did not

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detect constitutive OX2 expression in B10RII or C57/Bl6 mouse retina, although subsets of thymus and lymph node cell preparations expressed OX2 (data not shown). By flow cytometry, OX2 expression on resident myeloid-derived cells (CD45+CD11c+) was not as bright as in human or rat, although it was significantly four to five times greater than background isotype mAb controls (mean fluorescent intensity ± SD of 2.97 ± 1.1 background and 10.5 ± 2.3 OX2, n = 4).

**OX2 and OX2R Expression on Resident and Infiltrating Myeloid-Derived Cells during EAU**

Extent and distribution of expression of OX2 was studied during EAU in both rat and mouse. Because of sole availability of OX2 mAb (OX102) in the rat, flow cytometric and immunohistochemical data are presented in figures for rat only (Fig. 5). During the early stages of RE-induced EAU in the rat (day 8 after immunization), OX2R expression was detected by single-color immunohistochemistry on perivascular retinal and ciliary body cells (Figs. 5A, 5B). As myeloid infiltrate increased during peak EAU (day 14 after immunization), increased numbers of ED7+OX2R+ and OX12+OX2R+ cells were observed (Figs. 5C, 5D). Three-color flow cytometric analysis (Fig. 4) confirmed OX2R and OX2 expression in populations of CD45+CD11b/c+ cells. Figures 4A and 4B show dot plots of expression of CD45 and OX2 or OX2R on flow cytometrically gated CD11b/c+ cell populations, excluding granulocyte scatter. During EAU, OX2 was expressed on two populations of CD45hiCD11b/c−MG and CD45hiCD11b/c+ cells, respectively (Fig. 4A, arrows). Mean OX2R expression was three to five times greater than background and/or expression on the infiltrating T-cell population (plots in Figs. 4C, 4D, respectively), representing a real, albeit low, expression within the macrophage-monocyte cell population. Moreover, CD45high cells expressed OX2R more brightly (plot in Fig. 4B). It was more difficult to differentiate between MG and infiltrating monocyte-macrophages during EAU, because MG upregulate CD45 expression when activated in vivo. However, given that the whole population shift represented true expression of OX2R, although the majority of cells were infiltrating monocyte-macrophages, the population included resident MG. During IRBP peptide 1-20–induced EAU in

**FIGURE 2.** Comparison of cell localization of OX2 expression in human and rat retina by dual immunohistochemistry and immunofluorescence. Provisional work optimized detection of dual-positive cells, and the most efficient combination to maximize OX2 detection was by means of DAB immunohistochemistry or FITC immunofluorescence and the second primary antibody detected by APAAP and Texas red, respectively. MHC class II+OX2+ cells (arrows) by immunohistochemistry (A) and immunofluorescence (B) in human retina, respectively. Arrowhead: An MHC class II-positive cell. (C) An MHC class II+OX2+ perivascular cell within rat retina (arrow). Dual CD45−OX2+ cells were found in a perivascular location (D) and within the inner retina (E; arrows). Single-stained CD45+ cells were also found at this site (D; arrowhead). GFAP was expressed intensely throughout postmortem human retina, (F, G, and H) where single-color–positive OX2 cells are also visible (G, H, arrows). (I, J) Endothelial cells coexpressing OX2 (CD31−OX2+ cells; small arrows). Neuronal OX2 staining is visible in the surrounding parenchymal tissue (large arrow). Magnification, ×150–300.
C57/BL6 mice, three-color flow cytometric analysis detected an upregulation of OX2 expression on a subpopulation of CD45<sup>1</sup>CD11c<sup>1</sup> cells (MFI of 10.5 ± 2.1 normal to 21.5 ± 1.4 in EAU, n = 4) that was confirmed by immunohistochemistry.

**DISCUSSION**

After OX2R expression was described on macrophages, it was proposed that subsequent OX2–OX2R interaction may regulate macrophage activity. Although distribution of OX2 is widespread on endothelium of many organs, its constitutive expression on neurons within the CNS and eye may confer additional protection through regulation of OX2R<sup>+</sup> macrophage activity in organs, such as the posterior chamber of the eye, that are conditioned to regulate inflammatory responses. In this study, we have shown that within retina, OX2 is expressed in the main on GFAP-negative neurons and endothelium and on a subpopulation of CD45<sup>+</sup> myeloid-derived cells, although in the rat, CD45<sup>low</sup>CD11b/c<sup>+</sup> parenchymal MG were largely OX2 negative. mAbs used for different species have varying affinities for epitopes of OX2 antigen primarily because of differing methods of antibody production. Despite these

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**FIGURE 3.** Flow cytometric analysis of OX2 expression in human and rat retina. Figure represents composite of histogram profiles of fluorescence intensity of OX2 cell surface antigen expression. OX2 fluorescence was determined using three-color cell surface labeling to identify cell populations and analyzed in both CD45<sup>+</sup>CD11b/c<sup>+</sup> myeloid cells and CD45-negative cells (neuronal).<sup>14</sup> (A, B) Background fluorescence with isotype mAb controls. There was a two to three times mean fluorescence shift of OX2 expression on neurons (CD45-negative cells) in both human and rat (C, D, respectively), high peak (arrow) represents rod outer segment autofluorescence. (E, F) CD45<sup>+</sup>CD11b/c<sup>+</sup> cells expressing OX2. In rat (F), OX2-negative and low-expressing cells (arrow) were gated from CD45<sup>low</sup>CD11b/c<sup>+</sup> MG cells, and the OX2-positive population (arrowhead) was gated from the CD45<sup>+</sup>CD11b/c<sup>+</sup> perivascular cell population.<sup>14</sup>
differences, OX2 distribution and expression was comparable between rat and human (Figs. 1, 5). Figure 2 shows an unusual extent of intense staining of GFAP within human retina—particularly, in inner retina and end feet, cell bodies, and processes of presumptive Müller cells and astrocytes. In this study the extent of GFAP expression on glial elements was surprising. However, GFAP is upregulated when glia are activated under a variety of conditions.21–23 One interpretation is that GFAP upregulation is a nonspecific, leaky marker of activation analogous to the MHC class II upregulation observed with microglia in the eye and the CNS.24,25 Such findings of nonspecific activation are observed with increasing sampling delay after death. Despite the observed intense staining of GFAP, OX2 expression was confined to GFAP-negative neurons and not GFAP+ glial cells.

In mouse, although we were unable to detect OX2 expression by immunohistochemistry, flow cytometric analysis confirmed retinal cell expression that was upregulated during EAU. Preliminary data (C. Broderick, unpublished data, 2000) show that in OX2 gene knockout mice there is no abnormal retinal morphology, although resident myeloid-derived cells such as MG display an activated phenotype, expressing F4/80 and MOMA-2. Using mAb OX102 (mouse anti-rat OX2R), we were unable to identify OX2R expression by either immunohistochemistry or flow cytometry in resident myeloid cell populations, including MG in normal retina. However, during rat EAU, OX2R expression was observed on almost all CD45+CD11b/c+ monocyte-macrophage populations, representing both infiltrating leukocytes and MG during EAU.20 The distribution of OX2 and OX2R within normal retina and during EAU, respectively, suggests that the retina possess the potential to regulate macrophage activity.

The control of T-cell responses within CNS parenchyma, such as retina, remain undefined. Although no classic retinal dendritic cells have yet been identified, recent data infer that during mouse EAU, initiation of the inflammatory response may be secondary to the infiltration of dendritic cell populations18 and continued speculation that retinal MG are also APCs remains unconfirmed. In rat, only CD45R0+CD11b/c+ perivascular cells express OX2, and one notion is that mecha-
nisms such as those operating in protecting against allograft rejection are involved by modulating T-cell responses. A more likely mechanism is the in situ negative regulation of OX2R-positive myeloid lineage cells by OX2-expressing tissues, such as the retinal endothelium and neurons. In addition, OX2 expression may serve to constitutively regulate and maintain a steady state of resident macrophage activity within normal retina, which in turn downregulates OX2R expression and thus accounts for the absence of OX2R expression in normal retina.

During EAU, besides infiltration of the initiating antigen-specific T-cell infiltrate, antigen-nonspecific cells such as activated macrophages are pivotal for tissue destruction. For example, experiments have shown that in animals depleted of macrophages target organ destruction is suppressed. Furthermore, after neutralization of tumor necrosis factor (TNF-α) activity, a major proinflammatory cytokine, target organ protection is associated with a concomitant reduction in activated phenotype but not the number of infiltrating macrophages. We suggest that the constitutive expression of OX2 limits the tissue-destroying effects of macrophages and that investigation to ascertain under what circumstances and how macrophage-induced cytotoxicity and/or programming occurs within the retina is timely. For example, other preliminary experiments have supported current data that naive bone marrow-derived macrophages are programmed by exogenous interferon (IFN)-γ and TNF-α stimulation to produce nitric oxide (NO) and phagocytose. Furthermore, such programming is totally inhibited by pre- or simultaneous treatment with TNF-receptor fusion protein. During EAU, macrophages that have infiltrated the retina are rapidly programmed so that they are unable to be further manipulated by exogenous cytokines (Dick, unpublished data, 2000). Determining the role OX2R signaling plays in macrophage activation will assist not only in our understanding of control of inflammatory responses within the retina but also in the development of potentially novel therapeutic strategies.

References


