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## Establishment of a monoclonal antibody for human LXR $\alpha$ : Detection of LXR $\alpha$ protein expression in human macrophages

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### Abstract

Liver X activated receptor alpha (LXR $\alpha$ ) forms a functional dimeric nuclear receptor with RXR that regulates the metabolism of several important lipids, including cholesterol and bile acids. As compared with RXR, the LXR $\alpha$  protein level in the cell is low and the LXR $\alpha$  protein itself is very hard to detect. We have previously reported that the mRNA for LXR $\alpha$  is highly expressed in human cultured macrophages. In order to confirm the presence of the LXR $\alpha$  protein in the human macrophage, we have established a monoclonal antibody against LXR $\alpha$ , K-8607. The binding of mAb K-8607 to the human LXR $\alpha$  protein was confirmed by a wide variety of different techniques, including immunoblotting, immunohistochemistry, and electrophoretic mobility shift assay (EMSA). By immunoblotting with this antibody, the presence of native LXR protein in primary cultured human macrophage was demonstrated, as was its absence in human monocytes. This monoclonal anti-LXR $\alpha$  antibody should prove to be a useful tool in the analysis of the human LXR $\alpha$  protein.

### Background

Liver X activated receptor alpha (LXR $\alpha$ ) was first identified as an orphan member of the nuclear receptors expressed mainly in the liver [1,2]. LXR $\alpha$  is highly expressed in liver, intestine, kidney, spleen, Lung, and adipose tissue. LXR $\alpha$  requires retinoid X receptors (RXRs) as a partner to recognize and bind to its hormone response elements (HREs)

called LXRE, and regulates LXRE target gene expression in a ligand dependent manner. LXR $\alpha$  has been shown to be activated by a specific class of oxidized derivatives of cholesterol [3,4].

Previously, we compared LXR $\alpha$  mRNA expression in various internal organs using a DNA micro array and reported

the highest level of mRNA expression in human macrophages differentiated from human monocytes in the presence of GM-CSF [5]. LXR $\alpha$  regulates the expression of various genes in macrophages such as the ATP binding cassette transporters (ABCA1, G1 / G4 / G8) [6–10], apolipoproteins (ApoE / C-I / C-IV / C-II) [11,12], and lipoprotein lipase (LPL) [13] in macrophages. LXR $\alpha$  also regulates LXR $\alpha$  gene expression in macrophages [14–16].

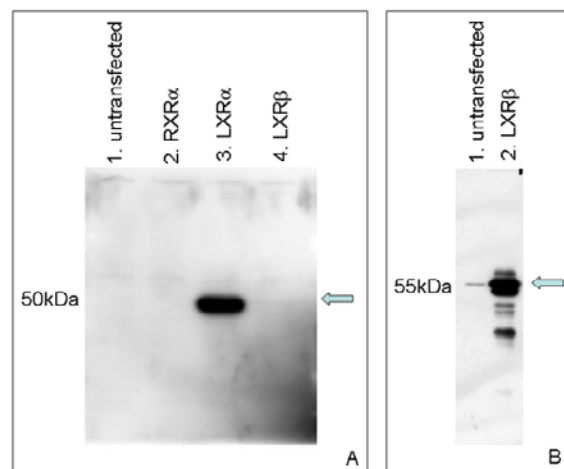
The structure and function of the LXR $\alpha$  protein has been studied in genetically engineered proteins or mammalian cell expression systems, but little information is available thus far on the physiologically expressed native protein. Rat liver LXR $\alpha$  protein has been studied by means of an antibody via immunoblotting [17,18] and electrophoretic mobility supershift assay [3,19,20], but analysis of the native human LXR $\alpha$  protein has not been carried out due to the lack of a sensitive anti-human LXR $\alpha$  monoclonal antibody.

Recently we have initiated a project designed to carry out a comprehensive analysis of the nuclear hormone receptors using a cluster of anti-nuclear hormone receptor monoclonal antibodies. Sensitive monoclonal antibodies against PPAR proteins and the RXR $\alpha$  protein helped complete an analysis of the native human PPAR proteins. We have also established a monoclonal antibody against the human LXR $\alpha$  protein, K-8607. Here we report the establishment and characterization of an anti-human LXR $\alpha$  monoclonal antibody. By means of this monoclonal antibody, native human LXR $\alpha$  protein in human monocyte-derived macrophage can be detected by immunoblotting. This antibody can be used for electrophoretic mobility supershift assay and immunostaining of COS-7 cells transfected with a human LXR $\alpha$  expression vector.

## Results

### Specificity of the anti-human LXR $\alpha$ mAb, K-8607

Figure 1(A) indicates the immunoblot analysis of the specificity of the mAb K-8607. Nuclear extracts were obtained from COS-7 cell transfected with human RXR $\alpha$ , LXR $\alpha$  or LXR $\beta$  expression vectors. Nuclear extract from untransfected COS-7 cells were used as a control. In each lane, nuclear extracts containing 20  $\mu$ g of protein were electrophoresed. The immunoblotting study indicated that K-8607 bound specifically to a 50 kDa protein expressed in COS-7 cells transfected with an LXR $\alpha$  expression vector. The apparent molecular weight of this protein is closely related to the calculated molecular weight of the human LXR $\alpha$  protein. The previously reported antibodies against LXR $\alpha$  have usually cross-reacted with LXR $\beta$  due to the high similarity of the primary amino acid sequence of both LXRs. In order to confirm the expression of LXR $\beta$  protein in COS-7 cells, immunoblotting analysis using mAb for human LXR $\beta$  K-8917 was performed. Figure 1(B)

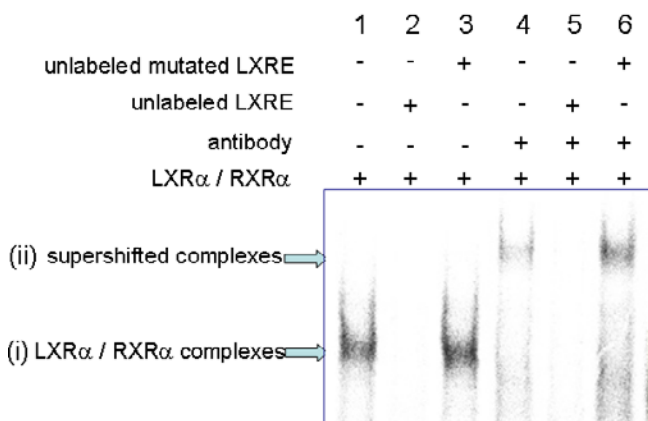


**Figure 1**  
**Immunoblot detection of LXR $\alpha$  in transfected COS-7 cells using anti-LXR $\alpha$  mAb and anti-LXR $\beta$  mAb**  
 Nuclear extracts of RXR $\alpha$ , LXR $\alpha$  and LXR $\beta$  transfected COS-7 cells (20  $\mu$ g per lane) were separated on a SDS-polyacrylamide gel (10%), subjected to electrophoresis and transferred to PVDF membrane. (A) Anti-LXR $\alpha$  mAb was used as first antibody. Lanes: 1, untransfected; 2, RXR $\alpha$ ; 3, LXR $\alpha$ ; 4, LXR $\beta$ . (B) Anti-LXR $\beta$  mAb was used. Lanes: 1, untransfected; 2, LXR $\beta$ .

indicates that a 55 kDa protein abundantly expressed in COS-7 cells transfected with an LXR $\beta$  expression vector and a small quantity of LXR $\beta$  protein was expressed inherently in COS-7 cells. These results indicate that K-8607 is specific to LXR $\alpha$  protein and does not recognize LXR $\beta$ .

### K-8607 supershift electrophoretic mobility of the DR4 oligonucleotide-nuclear extract protein complex

Figure 2 lanes 1 to 3 indicate the results of EMSA for synthetic DR4 oligonucleotide and nuclear extracts from COS-7 cells transfected with a human LXR $\alpha$  expression vector. DR4, which is the consensus binding sequence for human LXR $\alpha$ /RXR heterodimer complex, makes a complex with protein(s) in nuclear extract from COS-7 cells transfected with the LXR $\alpha$  expression vector (arrow 1). This complex was not detected when nuclear extract from untransfected cells was used (data not shown). The band disappeared upon addition of a 200-fold excess of unlabeled DR4 oligonucleotide, indicating that the binding is saturable (lane 2). The complex band did not change after the addition of a 200-fold excess of mutated LXRE oligonucleotide, which cannot bind to the LXR $\alpha$  / RXR heterodimer (lane 3).

**Figure 2**

**Electrophoretic Mobility Shift Assay (EMSA)** EMSA were performed with 10  $\mu$ g whole cell extracts and a 10 fmol [ $\gamma$ - $^{32}$ P] labeled probe. The specificity of binding to the respective probes was determined by using a 200-fold molar excess of unlabeled oligonucleotide as a competitor, which completely removed all the binding, indicating that these bound complexes resulted from sequence specific DNA-protein interactions. The arrows indicate the LXR $\alpha$  / RXR $\alpha$  complexes (i) and supershifted complexes (ii), respectively. Lanes: 1, LXR $\alpha$  / RXR $\alpha$ ; 2, with the addition of unlabeled DR4 oligonucleotide; 3, with the addition of unlabeled mutated LXRE oligonucleotide; 4, supershifted complex; 5, with the addition of unlabeled DR4 oligonucleotide; 6, with the addition of unlabeled mutated LXRE oligonucleotide.

Lanes 4 to 6 indicate the results of supershift assay with mAb K-8607. A higher molecular weight band (arrow II) appeared upon the addition of 10  $\mu$ g of mAb K-8607. This higher molecular weight band disappeared with the addition of a 200-fold excess of unlabelled DR4 oligonucleotide. This band was not affected by the addition of a 200-fold excess of mutated LXRE oligonucleotide. The original DR4-nuclear extract complex (arrow I) did disappear with the addition of mAb K-8607. These results indicate that mAb K-8607 is able to recognize the complex formed by DR4 and a component of nuclear extract from COS-7 cells transfected with LXR $\alpha$  expression vector. These results strongly suggest that mAb K-8607 is able to bind to the DR4/LXR $\alpha$  complex.

#### **Nuclear localization of mAb K-8607 antigen in COS-7 cells transfected with human LXR $\alpha$ expression vector**

Figure 3 depicts the result of mAb K-8607 immunostaining of COS-7 cells transfected with human LXR $\alpha$  expression vector. About one third of the transfected COS-7 cells exhibited pronounced staining (left panel). At higher magnification, the staining is clearly detectable in the nu-

cleus. COS-7 cells transfected with other expression vectors did not indicate this nuclear staining (data not shown). These results suggest that the antigen for mAb K-8607 is located within the nucleus. These results suggest the nuclear localization of human LXR $\alpha$  expressed in COS-7 cells.

#### **Detection of native human LXR $\alpha$ protein in human monocyte-derived macrophages by mAb K-8607**

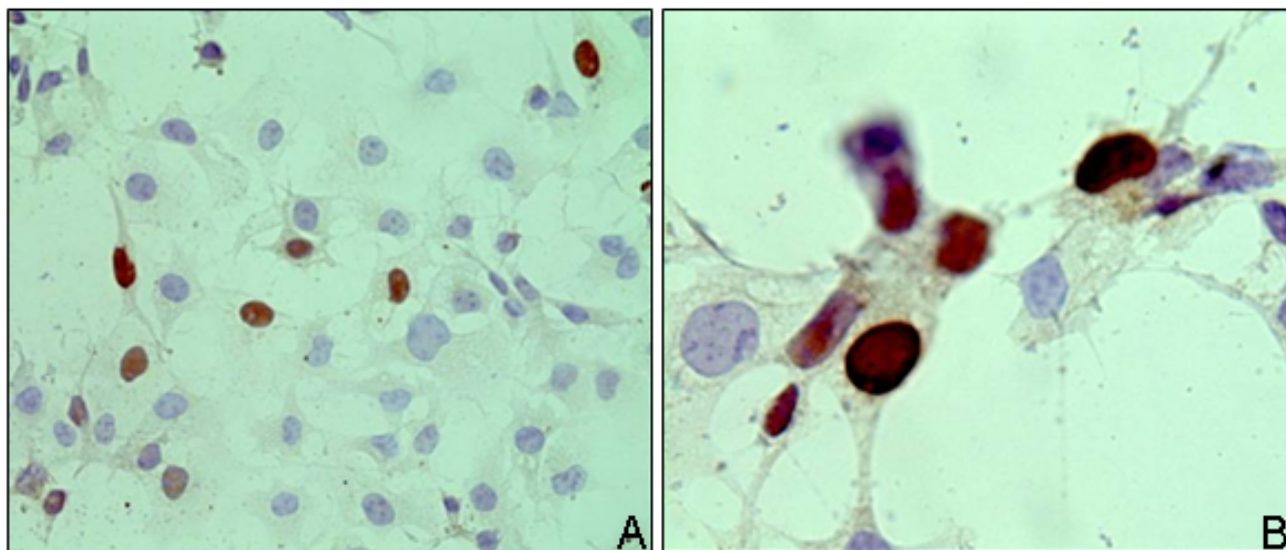
Figure 4 depicts the results of the immunoblotting study of human monocytes and macrophages using mAb K-8607. Previously our investigation had indicated that mRNA for LXR $\alpha$  is most abundantly expressed in human monocyte-derived macrophages. MAb K-8607 is able to detect human LXR $\alpha$  protein expressed in COS-7 cells. An identical molecular weight protein can be detected in human monocyte-derived macrophages. This protein was not detected in human monocytes, which express only a very small amount of LXR $\alpha$  mRNA. These results suggest that the 51 kDa protein detected by mAb K-8607 is native human LXR $\alpha$  protein. The amount of native protein in macrophages is significantly lower than in COS-7 cells expressing human LXR $\alpha$  protein.

#### **Discussion**

Analysis of the structure and function of the human LXR $\alpha$  protein to date has been performed mainly with genetically engineered proteins, since the analysis of native human LXR $\alpha$  protein has been hampered by the lack of a sensitive monoclonal antibody against the LXR $\alpha$  protein. The mAb K-8607 was established with an N-terminal 94 amino acid sequence in a baculovirus expression system. This monoclonal antibody recognizes a 51 kDa protein in nuclear extract from COS-7 cells, which is consistent with the calculated molecular mass (49,000) of the 447 amino-acid human LXR $\alpha$  protein.

A number of experimental results support the hypothesis that the mAb K-8607 antigen is human LXR $\alpha$ . The sequence similarity of the human LXR $\alpha$  and LXR $\beta$  proteins causes a specificity problem for establishing antibodies. In order to overcome this problem, we selected the N-terminal 94 amino acids. Within this region the similarity of the amino acid sequences is relatively low (35% identity). The results of immunoblotting indicated that mAb K-8607 is able to specifically recognize LXR $\alpha$ . Additional evidences further supports the specific recognition of the LXR $\alpha$  protein by mAb K-8607.

Immunohistochemical studies with COS-7 cells transfected with a human LXR $\alpha$  expression vector indicated that the antigen for mAb K-8607 is located in the nucleus. The intracellular localization of human LXR $\alpha$  has not been reported previously. Among the nuclear hormone receptors, several proteins are known to be expressed in the nucleus,



**Figure 3**  
**Immunohistochemistry of LXR $\alpha$  protein in LXR $\alpha$  transiently transfected COS-7 cells** Human LXR $\alpha$  transfected COS-7 cells were fixed in 4% paraformaldehyde in PBS for 10 min at room temperature. After fixation, the sections were treated as described previously.

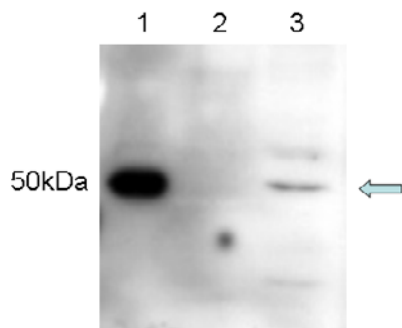
but some of the receptors, including the glucocorticoid receptor are expressed in the cytoplasm and ligand binding causes receptor translocation to the nucleus. In addition, the EGFP fused to N-terminal portion of human LXR $\alpha$  can be detected in the nucleus (Y. Watanabe et al manuscript in preparation). These results support LXR $\alpha$  protein localization in the nucleus.

Results of EMSA and supershift assay indicate that mAb K-8607 can cause supershift of the complex formed by the DR4 oligonucleotide binding sequence and nuclear extract protein from COS7-cells transfected with human LXR $\alpha$  expression vector.

We next studied the recognition of native human LXR $\alpha$  protein by mAb K-8607. We speculated that the difficulty of native LXR $\alpha$  protein detection may be caused by the relatively low level of native LXR $\alpha$  protein. We selected human monocyte-derived macrophages treated with GM-CSF for 7 days as the source of native protein in order to detect the native LXR $\alpha$  protein because this cell expresses the highest level of LXR $\alpha$  mRNA according to our previous investigations. The mRNA level in human monocyte-derived macrophages is highest among the 8 tissues and cells studied and the level is several fold higher than that in the liver. The result of immunoblotting indicated that even in monocyte-derived macrophages the level of the LXR $\alpha$  protein is very low as judged from the intensity of the immu-

noblot staining. In the case of the experiment shown in Figure 4, we applied 2  $\mu$ g of protein from COS-7 cells transfected with human LXR $\alpha$  expression vector but we needed to apply 75  $\mu$ g of monocyte-derived macrophage nuclear extract protein in order to detect the presence of the 50 kDa protein. In the case of the RXR $\alpha$  protein, ordinarily it is possible to detect the native RXR $\alpha$  protein with nearly the same amount of nuclear extract protein from COS-7 cells transfected with human RXR $\alpha$ . This result suggests that the level of native immunoreactive LXR $\alpha$  protein may be extremely low in human cells or tissues. We were not able to detect the native LXR $\alpha$  protein in human liver. This is not surprising because the mRNA level for LXR $\alpha$  in human liver is several fold lower than that in monocyte-derived macrophage. We were able to apply up to 100  $\mu$ g of liver nuclear extract protein for the immunoblotting assay, but this may not be sufficient for the detection of native protein from the result described here.

The reason the immunoreactive LXR $\alpha$  protein level is so low remains an open question, but previous difficulties with the study of the native LXR $\alpha$  protein are explainable based on this low protein level. LXR $\alpha$  is known to function as a heterodimer. In order to understand the delicate function of the LXR $\alpha$  protein in actual human cells, careful consideration and some novel technique will be needed to precisely assess the amount of the LXR $\alpha$  protein.



**Figure 4**  
**Immunoblot detection of LXR $\alpha$  in human monocytes and macrophages using anti-LXR $\alpha$  mAb** Nuclear extracts of monocytes and macrophages (75  $\mu$ g per lane) were separated on a SDS-polyacrylamide gel (10%), subjected to electrophoresis and transferred to PVDF membrane. Lanes: 1, Control; 2, monocytes; 3, macrophages.

Studies on the intracellular processing and/or degradation of the LXR $\alpha$  protein will be important future studies. The mAb K-8607 will be a critical tool for any such investigation.

### Conclusions

In summary, a mAb K-8607 was established which specifically detects human LXR $\alpha$  protein expressed in COS-7 cells or native human LXR $\alpha$  protein in monocyte-derived macrophages. The native human LXR $\alpha$  protein detected had an apparent MW of 50,000, which is close to the calculated 447 amino acids in the predicted LXR $\alpha$  protein. The preponderance of human LXR $\alpha$  protein in COS-7 cells is located in the nucleus. The expression level of native human LXR $\alpha$  protein is very low as compared with its heterodimeric partner, RXR $\alpha$ .

### Methods

#### Cell culture

Human primary monocytes / macrophages were obtained as previously described [5] and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS).

#### Establishment of antibody

Monoclonal antibody for human LXR $\alpha$  K-8607 was established as described previously [21]. Briefly, the N-terminal sequence of the human LXR $\alpha$  cDNA encoding amino acids 4–97 was inserted into a baculoviral transfer vector. Recombinant virus was produced and was purified and

then immunized. After ELISA screening mAb K-8607 was obtained. Monoclonal antibody for human LXR $\beta$  K-8917 was obtained by same method using the transfer vector inserted human LXR $\beta$  cDNA encoding amino acids 2–86.

#### Transient transfection

COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS. Cells were plated in a 100 mm dish at  $2.0 \times 10^6$  cells/dish for 16 hours prior to transfection. Transfections were performed by Effectene transfection reagent (QIAGEN) using 2  $\mu$ g of the pcDNA3-hLXR $\alpha$  expression vector.

#### Immunoblotting

Nuclear extracts were obtained as previously described [22]. Aliquots of each sample were separated on a 10% SDS-polyacrylamide gel and transferred to PVDF membrane. As a control for the correct LXR $\alpha$  protein band, we used a nuclear extract (2  $\mu$ g protein) of pcDNA3-LXR $\alpha$  transfected COS-7 cells.

LXR $\alpha$  proteins were immunochemically detected using mAb K-8607 (1  $\mu$ g/ml), and signal detection was achieved with a Super Signal West Dura Extended Duration Substrate.

#### Electrophoretic Mobility Shift Assay (EMSA)

EMSA was performed with nuclear or whole cell extracts from transiently transfected COS-7 cells and T4 polynucleotide kinase end-labeled oligonucleotides. 10  $\mu$ g nuclear extracts were incubated with 10 fmol of [ $\gamma$ - $^{32}$ P] labeled DR4 with or without a 200-fold molar excess of cold competitor oligonucleotide in a 15  $\mu$ l reaction in EMSA binding buffer (10 mM Tris-HCl pH 7.5, 50 mM KCl, 10 mM EDTA, 1 mM DTT, 1% Glycerol) for 30 min on ice. Supershift assays were performed by adding antibodies 30 min before or after incubation with an oligonucleotide probe. Protein-DNA complexes were resolved by electrophoresis on 4% polyacrylamide gel in  $0.5 \times$  TBE. Following electrophoresis, gels were fixed with 10% methanol / 10% acetic acid, transferred to moistened filter paper, dried by heating at 80 C under vacuum, and exposed to the imaging plate. The following double-stranded oligonucleotides were synthesized and used in the EMSA (sense strand shown): DR4, GATCTTAGTTCACCTCAAGTTCA-AGGATC; mutated LXRE, GATCTTGGTCCAGGCAAGTTCTAGGATC.

#### Immunohistochemistry

COS-7 and human LXR $\alpha$  transfected cells were fixed in 4% paraformaldehyde in PBS for 10 min at room temperature. After fixation, the sections were treated as described previously [21].

### Competing interests

none declared.

### Authors' contributions

Y. Watanabe carried out the molecular genetic studies and drafted the manuscript. T Tanaka, Y Uchiyama and T Takeno participated in the construction of the baculoviral transfer vector. A Izumi carried out the preparation of human monocytes. H Yamashita, J Kumakura and H Iwanari participated in the immunization. Jiang SY and M Naito participated in the immunohistochemical study. DJ Mangelsdorf provided the human LXR $\alpha$  expression vector for positive control. T Hamakubo participated in the study design of the study. Tatsuhiko Kodama conceived of the study and participated in its design and coordination.

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