Summary of Thesis for the degree of Doctor of Philosophy (Ph.D.)

Role of nuclear receptors in macrophage development and function

By

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1. INTRODUCTION

1.1.Nuclear receptors

1.1.1. Nuclear receptors: Introduction

Nuclear hormone receptors are ligand activated transcriptional factors that play important roles in the differentiation of tissues and organs and regulate several key metabolic processes. These receptors are located in the cytoplasm or in the nucleus depending on the species of the receptor and are activated by small lipophilic molecules that cross the plasma membrane or got generated inside the cell. After ligand binding receptors activate transcription of specific genes. Nuclear receptors make up a superfamily including classical steroid hormone, thyroid hormone (TR) and vitamin D receptor (VDR), retinoic acid receptors (RARs), receptors activated by intermediary metabolites: e.g. peroxisome proliferator-activated receptor (PPAR) by fatty acids, liver X receptor (LXR) by cholesterol metabolites and several orphan receptors. The term orphan comes from the fact that ligands of these receptors were unknown when the receptors were discovered. Several metabolites were identified later to activate these receptors but most of these have low binding affinity to the receptors. Nevertheless, identification of low affinity activators has led to the adoption of some orphan nuclear receptors (e.g. PPARγ, LXR). Members of this family share a conserved domain structure: 1. DNA binding domain (DBD) for anchoring the protein to specific DNA sequences, hormone response elements (HREs), 2. ligand-binding domain (LBD) for binding of small lipophilic molecules, 3. trans-activation domain for activating the basal transcriptional machinery.

Most nuclear receptors function as dimers. Classical steroid hormone receptors like estrogen receptor (ER), androgen receptor (AR), glucocorticoid receptor (GR) form homodimers. The non-classical nuclear receptors like RARs, VDR, TR are localized in the nucleus sitting on their hormone response elements in the enhancers of target genes and most receptors repress transcription. Upon ligand binding a conformational change occurs that results in the release of co-repressor complex and recruitment of co-activator complex leading to transcription of target genes. Retinoid X Receptor RXR was shown to form heterodimers with many other nuclear receptors. The homo- or heterodimers recognize specific DNA sequences (hormone response elements): direct, indirect or inverted repeats.

1.1.2. Nuclear receptors: Retinoid receptors

Vitamin A and its derivatives, retinoids have profound effects in development, differentiation, homeostasis and various aspects of metabolism. The discovery of retinoid receptors substantially contributed to the understanding how these small, lipophilic molecules, most importantly retinoic acid (RA) exert their pleiotropic effects.

The two families of retinoid receptors (RARs and RXRs) now contain three isotypes, α, β and γ encoded by separate genes and giving rise to numerous alternatively spliced variants. RARs can be activated by all-trans retinoic acid (ATRA) and 9-cis RA, while RXRs can be activated only by 9-cis RA. RARs have been implicated in embryonic, skeletal, myeloid development, wound healing, keratinization and also in the developing nervous system. RXR is unique among nuclear receptors because in addition to forming homodimers it forms many heterodimers and therefore ligand activation has potentially pleiotropic effects on numerous biological pathways and has been implicated not only in retinoid responses but also in various metabolic pathways. The expression pattern of the subtypes is rather different. RXRα shows abundant expression in liver, kidney, spleen, placenta, epidermis and a variety of visceral tissues, RXRβ is expressed widely and can be found in almost every tissues (like RARα), RXRγ expression is mainly restricted to muscle and brain.

1.1.3. Nuclear receptors: PPARs

PPARs are involved in the regulation of important metabolic pathways, most of them play critical role in fatty acid and cholesterol metabolism. The family contains three isoforms: PPARα, γ and δ. PPARα is mostly expressed in brown adipose tissue and liver, then kidney, heart and skeletal muscle. PPARγ is mainly expressed in adipose tissue, macrophages and to a lesser extent in colon, in the immune system and the retina. PPARδ is found in many tissues, ubiquitously expressed but the highest expression is in the gut, kidney and heart. The function of the PPARs have been extrapolated from the identity of their target genes, which so far all belong to pathways of lipid transport and metabolism. PPARγ controls a broad range of cellular responses: differentiation, proliferation, cell death and inflammation. It is essential for the development of adipose tissue, plays critical role in glucose homeostasis. In mice it is essential for placental development and vascularization. PPARγ can be activated by natural ligands such as fatty acids, oxidized

low density lipoprotein (oxLDL) component lipids, 11, 13-hydroxy octadecadienoic acid, 15-deoxy-d-(12,14)PGJ2 (15D-PGJ2) and the thiazolidinedione class of insulin-sensitizing drugs that have been developed and used in the treatment of type two diabetes mellitus. PPARγ gene knockout resulted in early embryonic lethality. Deficiency interferes with terminal differentiation of the trophoblast and placental vascularization, leading to severe myocardial thinning and death by E10.0. The lack of the PPARγ gene in macrophages is likely to be pro-atherogenic.

1.1.4. Nuclear receptors: Liver X receptors

Two LXR proteins (α and β) are known to exist in mammals. LXR also function as heterodimer with RXR. Expression of LXRα is restricted, with highest levels in the liver and lower, but significant levels in macrophage, kidney, intestine, spleen and adrenals. LXRβ expression is more ubiquitous and has been found in nearly every tissues examined. Several oxysterols, like 22(R)-OH cholesterol 25-OH and 27-OH cholesterol have been identified as ligands for LXR. LXRs have been implicated in the regulation of cholesterol metabolism and clearance and recently in inflammation. Mice lacking LXRα lose their ability to respond normally to dietary cholesterol and unable to tolerate any amount of cholesterol in excess of that they synthesize de novo. These mice develop severe atherosclerosis.

1.2. Monocytes-macrophages

1.2.1. Monocytes-macrophages/Introduction

Monocytes and polymorphonuclear phagocytes develop from pluripotent stem cells characterized by the expression of CD34. These cells give rise to granulocyte-monocyte, granulocyte- and monocyte-colony forming units (CFU-GM, CFU-G and CFU-M). Monocyte differentiation proceeds from CFU-M through monoblast to circulating monocytes, which mature to macrophage in various tissues leading to the formation of multiple types of tissue-specific macrophages {Friedman, 2002 #1}. Markers for this lineage are M-CSF receptor, lysozyme, macrosialin and cell surface proteins (e.g. CD36, CD14, CD11b and CD18). The differentiation of myeloid cells is principally regulated by cytokines and orchestrated via cooperative gene regulation by various transcription factors. RAR was reported to act as a differentiation checkpoint switch at the promyelocytic stage of granulopoiesis resulting in granulocytic differentiation. Retinoic

acid (RA) stimulates the maturation of myeloid precursors in cytokine-stimulated CD34 positive cells. The 15;17 chromosome translocation in acute promyelocytic leukemia (APL) generates a PML-RARα fusion protein that inhibits RARs, resulting in a block of terminal differentiation of granulocytes.

PPARγ was shown to influence myeloid development. PPARγ has not appeared to regulate the formation of the monocytic lineage but modulates differentiation and metabolic functions of macrophages. Recent observations suggest that although PPARγ is not absolutely necessary for monocyte differentiation, modulation of the level and activity of PPARγ has critical consequences in the fate and metabolism of a macrophage. The mechanism by which PPARγ affects differentiation is still unknown and the possible interconnection between retinoid and PPARγ signaling in the maturation process has not been studied in detail yet.

1.2.2. Monocytes-macrophages/Lipid metabolism – Atherosclerosis

Atherosclerosis is a progressive, degenerative disease of blood vessels. Initially, it is characterized by lipid accumulation in the wall of arteries. The formation of the atherosclerotic lesion begins after birth and clinical manifestations can be observed usually from the sixth decade. Complications of atherosclerosis such as myocardial infarction and stroke are responsible for most of the deaths in western societies. This fact gives particular importance and relevance to studies on the formation of atherosclerotic lesions and to the efforts to identify molecular targets for potential anti-atherogenic drugs. Handling of lipids by macrophages is an important metabolic process in the context of hypercholesterolemia and the development of atherosclerotic lesions. Preferred sites of lesions are where endothelium is vulnerable. Endothelial dysfunction results in the accumulation of low-density lipoprotein (LDL) in the sub-endothelial matrix. It is not clear how LDL gets modified but this modification leads to the appearance of minimally oxidized/modified (mmLDL) and subsequently fully oxidized LDL (oxLDL) containing multiple oxidized lipid molecules. Uptake of the modified LDL and oxidized lipids is not complete so they are able to modulate the function of the neighboring cells, which lead to production of inflammatory cytokines by multiple cell types such as endothelial cells, lymphocytes and monocytes. The release of these inflammatory mediators evokes a characteristic inflammatory response around lipid accumulation by attracting additional

monocytes to the lesion and causes their maturation into macrophages. The macrophages accumulate lipids from oxLDL leading to lipid filled foam cell formation. Foam cells are the characteristic cells of the early, cellular phase of atherosclerotic lesions formation, they eliminate lipids from the sub-endothelium but unable to get rid of all of it if overwhelmed so they accumulate lipids continuously and this contributes to a sustained chronic inflammation that make smooth muscle cells migrate from the vessel wall's media, proliferate and produce extracellular matrix. This leads to the formation of the late, fibrous atherosclerotic plaques. This late lesion is characterized by sclerosis, which makes the artery wall rigid and fragile. Finally, the lesion may rupture the endothelium covering it leading to the formation of thrombus and intravascular coagulation.
PPARγ was shown to promote uptake of oxidized LDL and subsequent differentiation of the monocytes to foam cells. Synthetic PPARγ agonists caused similar differentiation both in myelomonocytic cell lines and in primary human monocytes, too. It was shown that PPARγ was expressed in foam cells of atherosclerotic lesion. Its expression could further increased with oxLDL. Thus, oxLDL but not native LDL promoted uptake of itself by scavenger receptor CD36. Characterizing this phenomenon it was proved that PPARγ had a key role in the regulation of lipid uptake into foam cells. Briefly, the scavenger receptor CD36 could be regulated by PPARγ as a direct target gene of the receptor. Two components from the lipids in oxLDL, 9-hydroxy octadecadienoic acid (9-HODE) and 13-HODE were identified as endogen activators and *bone fide* ligands of PPARγ. On the contrary, oxLDL increase the expression of a scavenger receptor CD36 by PPARγ and potentiates its own uptake. The model for foam cell formation referred to as gamma cycle above put PPARγ on the dark side of atherogenesis, suggesting its activation may be pro-atherogenic. However, synthetic agonists of PPARγ, the thiazolidinediones (TZDs) are widely used in treatment of type II diabetes. The question, how PPARγ regulates atherosclerosis remained unclear. Is its activation beneficial or harmful for the patients with atherosclerosis or diabetes? PPARγ was shown to not only regulate lipid uptake, but had a central role in cholesterol efflux from cells. PPARγ induces ABCA1 expression and cholesterol removal from macrophages through a transcriptional cascade driven by PPARγ and LXRα. ABCA1 and ABCG1 are members

of the ATP-binding cassette family of transporter proteins. Several studies reported that LXRs mediate cholesterol efflux by inducing cholesterol transporters like ABCA1, ABCG1. Cholesterol efflux is likely to be anti-atherogenetic. The effect of oxLDL to induce cholesterol efflux from macrophages is also anti-atherogenic, but as described above oxLDL promotes a potential vicious circle activating PPARγ and enhancing its own uptake to the macrophages. This contradiction was resolved by showing that activation of PPARγ leaded to primary induction of LXRα. Tontonoz and colleagues analyzed the promoter of the ABCA1 gene and showed that LXR:RXR could activate it. So, PPARγ induces the expression of LXRα, which activates transcription of cholesterol transporters e.g. ABCA1 and these lead to increased cholesterol efflux to ApoAI from macrophages. Linking of the two receptor systems (PPARγ and LXRα) provides an attractive but not well understood model to explain lipid/cholesterol uptake and efflux from macrophages. The issue how the activation of the two receptors may be coupled has not been addressed yet. It was assumed that lipid content of lipoproteins may act as activators or ligands for both PPARγ and LXR. The fact that LXR signaling is activated in macrophages exposed to acetylated LDL, which does not contain oxidized cholesterol suggests that there must be other ways to activate/produce ligand for this receptor. PPAR-related induction of LXRα is not enough for getting an activated LXR that induces cholesterol efflux. It needs to be activated by its endogenous ligand. Production of endogenous LXR activator that itself induces changes through LXR has not been reported yet. Furthermore, LXR:RXR heterodimers were originally identified as mediators of an alternative retinoid signaling pathway showing that LXR:RXR heterodimer is highly permissive and can be activated from either the RXR side by retinoids or the LXR side by oxysterols. A number of oxysterols were identified as potential endogenous ligands for LXR. One of these compounds, 27-hydroxycholesterol is produced by a p450 enzyme CYP27. CYP27 was reported to be expressed besides the liver in the lung and also in macrophages and also in atherosclerotic lesions. A mutation in this enzyme leads to a human disease cerebrotendinous xanthomatosis (CTX), a rare sterol storage disease characterized by xanthomas in tendons and CNS leading to ataxia, spinal cord paresis, neurological dysfuntions, xanthomatosis and atherosclerosis. The enzyme's product 27-hydroxycholesterol has been shown to activate LXR. These data raised the possibility

that CYP27 might serve as a regulator of LXR activity by generating ligand to it.

2. SUMMARY AND QUESTIONS REMAIN TO BE ANSWERED

Many aspects of nuclear receptors' function in macrophages have been studied. As a conclusion one can see that there are predominant receptors in macrophages: RAR (mainly α and γ), RXR (mainly α and β), PPAR (mainly γ) and LXR (mainly α). The major processes regulated by RARs are likely to be related to differentiation. Little is known about RXR's role, though. It can be linked to RARs, PPARs and LXRs but there are pieces of evidence that indicate the existence of separate RXR activity. This obviously needs to be further characterized. Most data from recent years show that PPARγ is a key nuclear receptor in macrophages. PPARγ is the regulator of another nuclear receptor, LXR in these cells and besides the anti-inflammatory effects of its ligands PPARγ was also tied to an important macrophage function, the clearance of oxidized cholesterol. A cycle has been described with PPARγ in the center coordinating lipid traffic into and from the cells. Once lipids from oxLDL have activated PPARγ it leads to a feed-forward regulation and increased expression of scavenger receptors that leads to further oxLDL uptake. Second, it induces LXRα which if becomes activated induces reverse cholesterol transport, cholesterol efflux from the cells.

However, several questions remain to be answered. It was shown that activation of PPARγ leads to the induction of maturation markers but PPARγ *per se* is not necessary for macrophage differentiation. It is clear from other studies that RARs regulate myelopoiesis. How are these two receptors interconnected, if at all?

PPARγ was shown to be activated by oxidized fatty acid coming from oxLDL. What about LXR? How is it activated? Where does the ligand come from? LXR:RXR is also a highly permissive heterodimer. What is the role of retinoids in LXR activation?

From a functional point of view macrophages are heterogeneous. Does the activation state affect nuclear receptor regulated processes? If yes, how?

These are the questions we set out to answer.

3. AIMS

We wanted to analyze three potential interactions in details: 1. RAR-PPAR crosstalk in myeloid differentiation, 2. PPARγ-LXR crosstalk in macrophage lipid metabolism and 3. relationship between macrophage activation and PPARγ response.

1. Crosstalk between RARs and PPARs

RAR was reported to act as a differentiation checkpoint switch at the promyelocytic stage of granulopoiesis resulting in granulocytic differentiation as detailed above but recently it was more generally connected to myelopoiesis suggesting a role in the early common steps of granulopoiesis and monopoiesis and has been also implicated in later stages of monocytic development. We wanted to characterize the expression of PPARγ in various stages of development and analyze if retinoids modulate the maturation process and/or PPAR activity, PPAR-mediated maturation.

2. Crosstalk between PPARγ and LXR

PPARγ induces transcription of LXRα. But how is LXR activity regulated? Is it constitutively active by a huge excess of endogenous activators or is there a need for the synthesis of endogenous agonist? Our preliminary data suggested that endogenous ligand synthesis was required and we wanted to find how this happens and if it is regulated by PPARs or other nuclear receptors. First we identified similarly expressed genes in macrophages and compared these to the ones regulated by PPARγ. By global gene expression profiling we were able to find potentially interesting genes in ligand production that were further characterized.

3. Role of macrophage activation state in gene expression

Last but not least we were interested in the type of macrophages where PPARγ and/or LXR are present and active. Several studies have been reported about nuclear receptor-regulated processes so far but none of these analyzed nuclear receptor activity from the macrophage point of view. Therefore we differentiated monocytes to macrophages and activated these cells with various agents (cytokines or pathogen derivatives) to compare the expression and function of nuclear receptors implicated in lipid metabolism to determine if the activation state of the cells can add a third dimension to the regulation of receptor activity besides the presence of receptors and the presence/production of their ligands.

2. RESULTS

2.1. Retinoids potentiate PPARγ response in differentiation, gene expression and lipid metabolic processes in developing myeloid cells

2.1.1. PPARγ is the dominant receptor in macrophage development

PPARγ is the most highly induced nuclear receptor in differentiating myeloid cells and its expression increases parallel with the degree of maturation. Moreover, activation of PPARγ contributes to subsequent differentiation in the monocytic pathway. We have also found a crosstalk between retinoid and PPARγ signaling: retinoids potentiate developing cells' response to PPARγ activators. This crosstalk represents a novel convergence of the two signal pathways important in the maturation of myeloid precursors and suggests new opportunity for regulating PPARγ-related metabolic processes.

First we compared the expression of PPARα, γ and δ in primary human CD34 positive cells after isolation and following differentiation with M-CSF for eight days to primary human macrophages. Surprisingly, we found a significant difference in the mRNA expression profile in the various conditions. During the maturation of stem cells we could detect a 7-fold induction of PPARγ with minor increase in PPARα and δ levels. Similarly, in the monocyte/macrophage transition PPARγ induction was the highest (27-fold). As a model of the different stages of myeloid differentiation we chose myeloid leukemia cell lines: KG-1 representing acute myelogenous leukemia, HL-60 representing acute promyelocytic leukemia (FAB M3), THP-1 (FAB M5) and MonoMac-6 (FAB M5) representing two monocytic leukemia cell lines. MonoMac-6 proved to be most committed to the monocytic lineage characterized by its increased phagocytic capacity, chemotactic potential, cytokine production and cell surface expression of monocytic markers. PPARs are expressed at high levels in these cell types except the least matured KG-1 cells. PPARα level is similar in the other three cell lines, PPARδ is higher in the two monocytic leukemia cell lines and PPARγ mRNA is the most abundant in the most matured MonoMac-6 cells reaching the level of that of the macrophage's. These data suggested that monocyte/macrophage differentiation was accompanied by an induction in PPARγ and at a lesser degree of PPARδ levels. The significant induction in PPARγ levels raised the possibility that activation of the receptor may be part or contribute to the

maturation process. Expression of retinoid receptors did not show such a change during maturation but their presence indicated possible roles for these receptors in macrophage development.

2.1.2. PPARγ activation leads to monocytic maturation

In the least differentiated cell line (KG-1) PPARγ activation resulted in no significant changes in cell surface markers (data not shown), which was probably due to the very low level of the receptor expressed in these cells. In the most matured MonoMac-6 cells activation of the PPARγ:RXR heterodimer with synthetic agonists, Rosiglitazone for PPARγ and LG268 for RXR, induced the expression of CD14, CD36 differentiation markers on the cell surface. These data suggested that activation of PPARγ:RXR heterodimer enhanced differentiation of cells in the monocytic pathway. There is extensive evidence suggesting that retinoids play important roles in the early developmental processes of hematopoietic cells. We tested whether they induced myeloid differentiation. On one hand we found that RAR agonists caused maturation only in the least differentiated cells (KG-1, HL-60) examined directing them towards the granulocytic lineage but we have found no evidence that retinoids could induce monocyte/macrophage differentiation in these cell lines (data not shown). On the other hand, RXR specific agonists caused similar effects than PPARγ activators, suggesting that RXR specific ligands might activate and function through PPARγ:RXR heterodimers.

2.1.3. Retinoids enhance PPAR response

Very surprisingly, combination of RAR and PPARγ activators resulted in an unexpected synergy on PPARγ induced gene expression. In the experiments we used sequential treatment (retinoids followed by PPARγ activators), because we reasoned that it probably represented a more physiological setting (i.e. previous reports propose a role for retinoids in the earlier steps of myeloid cell differentiation). When we treated the MonoMac-6 cells sequentially first with RAR agonists and then with PPARγ agonists we observed an increased effect of PPARγ specific ligands on gene expression of CD14 and CD36. We characterized this retinoid-evoked potentiation of PPARγ response by measuring the induction of various PPARγ target genes: fatty acid-binding protein 4 (FABP4), CD36,

adipose differentiation-related protein (ADRP) and PPARγ angiopoietin-related protein (PGAR). Rosiglitazone readily induced target gene expression in MonoMac-6 cells in the cases of FABP4 (x42), CD36 (x5) and PGAR (x150) and in THP-1 cells in the case of FABP4 (x43) and PGAR (x3). THP-1 cells behaved similarly. 9-cisRA and AM580 were similar to ATRA. The highest inductions could be observed in the case of AM580+LG268 pretreatments. These results clearly indicated that retinoid pretreatment although differentially affected the two cell types and the four target genes as a general rule potentiated PPARγ responses. A significant component of this potentiation is induced by RARα selective retinoids. Obviously, there are many potential mechanisms to account for this effect ranging from epigenetic changes to changes in cofactor and receptor levels. We tested the most obvious, the levels of PPARs. We measured PPAR mRNA levels and found that both natural and synthetic RAR and RXR agonists induced PPARγ mRNA levels while PPARα and δ mRNA levels were unaffected in both THP-1 and MonoMac-6 cells. Finally, we sought to obtain evidence for the biological consequence of retinoid enhanced PPARγ response. Therefore, we measured the uptake of diI-labeled oxLDL in retinoid-pretreated MonoMac-6 cells after activation with PPARγ agonists. Retinoid-treated cells and only those that were treated with RAR agonists became capable of taking up oxLDL after PPARγ agonist treatment alone. These results showed that retinoid pretreatment not only induced increased transcription of PPARγ target genes but also facilitated oxLDL uptake into the cells providing a potentially new target for the modulation of cholesterol-eliminating machinery.

2.2. Transcriptional regulation of human CYP27 integrates retinoid, PPAR and LXR signaling in macrophages

2.2.1. Human CYP27 is acutely regulated by retinoids and PPARγagonists

We have carried out DNA microarray analysis on monocytic leukemia cells (MonoMac-6) treated with PPARγ (Rosiglitazone) and the RXR selective retinoid LG268 and also on primary human monocytes induced to differentiate to macrophages. We found that transcript levels of the p450 enzyme CYP27 increased in monocyte-derived macrophages and also in PPARγ and RXR ligand treated myeloid cells along with several other genes associated with lipid metabolism (data not shown) suggesting that these genes may be

under PPARγ and/or retinoid regulation. We determined the transcript levels of various nuclear receptors (PPARs, retinoid receptors and LXR) during monocyte-macrophage transition and found an increased PPARγ and LXRα level. Strikingly, it was paralleled by an increase in CYP27 transcript levels, the enzyme with the potential to produce a soluble cholesterol metabolite and a ligand for LXRα.

It was intriguing to speculate that there was a link between the well-established PPARγ-LXR signaling and induction of CYP27 in macrophages. Therefore, we decided to determine if ligand activation of nuclear receptor pathways could activate CYP27 gene transcription.

2.2.2. RAR, RXR and PPARγ induce CYP27

Using monocyte-derived macrophages and the monocytic leukemia cell line (MonoMac-6) we tested the effect of RAR, RXR, PPAR and LXR specific ligands on the expression of CYP27. Retinoids and activators of PPARγ could induce the transcript levels of CYP27 to high levels in human macrophages. Compounds with RAR selectivity (such as all trans retinoic acid (ATRA), AM580) and also one with RXR selectivity (LG268) proved to be effective inducers and we could detect an increased induction upon treatment with the PPARγ specific Rosiglitazone in combination with the RXR specific LG268. These experiments strongly suggested that RAR:RXR and PPARγ:RXR heterodimers can transcriptionally regulate the human CYP27 gene.

2.2.3. RAR:RXR and PPARγ:RXR heterodimers bind to and activate the human CYP27 promoter

Our findings prompted us to look at the previously defined promoter region of human CYP27. Using an 853 bp fragment we carried out transient transfection experiments asking if receptor heterodimers can activate this promoter. Co-transfection of RAR and RXR receptors and addition of 9-cis retinoic acid (9-cisRA) induced a more than 5-fold increase in promoter activity. Similarly, co-transfection of PPARγ and RXR in the presence of both PPARγ and RXR specific ligands increased transcriptional activity. These results indicated that the 853 bp fragment contained all the necessary information to mediate both retinoid and PPARγ-dependent regulation. To define the response elements we looked at various deletion mutants and found that the detected induction was

approximately half of that of the 853 bp promoter fragment when a shorter fragment was used (649 bp). An additional deletion (217 bp fragment) led to retained core promoter activity and to the complete loss of inducibility. These experiments suggested that more than one response element may be localized between -853 and -217 bp. In silico analysis of the promoter identified two regions. We termed these PRRE (Peroxisome Proliferator Activated Receptor–Retinoid Response Elements) A and B. The A element contained an unusual arrangement of binding sites, three binding sites arranged in an overlapping DR1-DR5 configuration (i.e. two direct repeats sharing a half site). The B element looked simpler, it was a direct repeat separated by one nucleotide (DR-1).

2.2.4. Characterization of a response element mediating retinoid and PPARγ signaling

In order to test if the identified element could indeed bind receptor heterodimers *in vitro* we carried out Electrophoretic Mobility Shift Assays (EMSA). It is shown that the major element (hCYP27-PRRE-B) has a DR-1 arrangement. EMSA analysis revealed that this DR-1 is able to mediate both PPARγ:RXR and RAR:RXR binding. The specificity of binding was demonstrated by the use of antibodies against RXR and PPARγ inducing supershifts or reduced binding, respectively. A similar set of experiments were carried out using RAR:RXR heterodimers. RAR:RXR and PPARγ:RXR can bind to the same element utilizing a DR1. By using VP16 fusion receptors (ligand mimic receptors) and transient transfection we could show a strong and robust binding of the PRRE-B with VP-RXR and VP-RXR and PPARγ. To prove that the identified response element is a functional enhancer, enhancer trap vectors were constructed fusing two copies of the element to a minimal TK promoter and a luciferase reporter gene. Based on these data the B element (hCYP27-PRRE-B) appeared to be critical in mediating transcriptional activation with a potentially minor contribution of a weak, accessory element (hCYP27-PRRE-A).

2.2.5. CYP27 expression results in 27-hydroxycholesterol formation and efflux

We carried out mass spectrometric determination of the metabolic products of CYP27 from cell pellets and media supernatants. The determined compounds were 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid. ATRA and 9-cisRA as well as

synthetic ligand for RARα (AM580) or RXR (LG268) induced 27-hydroxycholesterol formation and release to the medium.

2.2.6. 27-hydroxycholesterol activates LXR and induces target genes

LG268 and 9-cis retinoic acid synergizes with 27-hydroxycholesterol to induce gene expression to a very significant degree. 27-hydroxycholesterol at 10 □M concentration was able to induce a 14-fold induction whilst if combined with 9-cisRA the induction was further induced with an additional 19-fold resulting in a nearly 300-fold induction altogether. These data suggest that retinoids via the induction of a partial agonist of LXR gain competence to induce robust LXR-dependent transcription.

2.2.7. Retinoids induce LXR-mediated gene expression via activation of CYP27

Retinoids and LXR ligand induced ABCA1 and ABCG1 to varying degrees. The induction of ABCA1 and ABCG1 by RXR or pan-retinoid agonist ligands is not unexpected since they are believed to regulate LXR:RXR heterodimers from the RXR side, but the RAR selective ligand AM580 could also induce ABCA1 and ABCG1. Combination of RAR and RXR ligands were slightly better than the RXR ligand alone. Our findings therefore suggest that retinoid induction of LXR target genes could involve activation of CYP27 and production of 27-hydroxycholesterol. We used primary cell lines derived from patients with CYP27 deficiency (CTX). We could demonstrate that retinoids induced CYP27 expression not only in myeloid cells, but also in fibroblasts and that retinoid induced ABCA1 expression is strongly attenuated in the absence of CYP27 (in CTX fibroblasts).

2.2.8. Retinoid and PPARγ regulated gene expression exist in human atherosclerotic lesions

We analyzed the gene expression profile of human macrophage rich atherosclerotic lesions and a remarkable increase of PPARγ, LXRα, their target genes ABCA1, CD36 and CYP27 was detected if compared to normal vessel wall. These data suggest that lesion tissue (mostly macrophages) have an expression profile similar to that of *in vitro* differentiated macrophages.

2.3. PPARγ responsiveness in differently activated macrophages

2.3.1. PPARγ is highly expressed in alternatively activated macrophages

Once monocytes enter the subendothelial tissues they are transformed to macrophages and become activated by extracellular signals to fulfill their task. Basically, there are two forms of macrophage activation: the classical one which is induced by inflammatory agents and cytokines and results in an inflammatory macrophage and an alternative activation pathway is also exists that was found to be an inhibitory, anti-inflammatory process induced by mainly interleukin-4 (IL4) but recently many positive consequences of alternative macrophage activation have been described e.g. roles in antigen presentation, resolving the inflammation, clearance of tissue debris, phagocytosis and repair. We wanted to test if the state of macrophage activation affects/correlates with the expression and function of PPARγ. Therefore, we activated monocyte-derived macrophages in vitro by using INFγ+TNFα for the classical and IL4 for the alternative activation. The activation state of the macrophage determines the level of PPARγ whilst other PPARs does not show any differences. PPARγ is highly induced upon IL4 treatment. There is a major difference in the PPARγ responses in the differently activated cells. PPARγ target genes can be differently induced in the differently activated cells. Nevertheless, target genes were inducible only in the alternatively activated macrophages and to a minor degree in the non-activated cells. This cannot be explained with the different PPARγ levels, further characterization is needed to find this an unknown mechanism. We have also analyzed PPARγ expression and responses in murine macrophages. We used resting peritoneal macrophages, thioglycolate-elicited macrophages and bone marrow-derived macrophages. These cells are different from the human ones, but they resemble the human macrophages leastwise in the pattern of PPAR expression. We performed microarray experiments from the human macrophages to see if the enhanced PPARγ responsiveness a general phenomenon is or is limited for a subset of genes. Interestingly, we found that more gene were upregulated in the alternatively activated macrophages than in the control or classically activated cells.
Furthermore, not only the number of genes induced were higher but those genes that were induced in at least two conditions were expressed at higher levels shown by the absolute signal values. The most exciting observation was that only a few genes were regulated in at least two cell types and none in all the three conditions. These results clearly show that alternatively activated macrophages are the main cells where PPARγ is expressed and

active.

3. DISCUSSION

3.1. Retinoid-PPAR crosstalk

After the initial discovery of PPARγ in myeloid cells there have been several studies trying to define its biological role. Many studies including some of our own has established PPARγ as a differentiation related transcription factor in myeloid cells. However, no systematic analysis of its expression and the regulation of the PPARγresponse have been carried out on myeloid cells of human origin.

In this study we have attempted to systematically characterize the level of PPARγ in human normal and leukemic myeloid cell. We have established a role for nuclear receptors in myelogenous differentiation, by showing that PPARγ expression is tightly linked to the differentiation stage of myeloid cells in the monocytic lineage and that promotion of differentiation induces the expression level of this gene. Moreover, activation of this nuclear receptor results in the induction of differentiation markers of macrophages. We also showed that retinoids potentiate the effects of PPARγ activators by inducing the transcription of PPARγ itself. We provided evidence for that retinoids may contribute to the physiological/pathophysiological function of PPARγ by increasing the uptake of oxLDL particles. These findings establish a link between retinoid receptors and PPARγ in myeloid differentiation and implicate RAR as a potential "jump-starter" of the non-established PPARγ signal pathway in macrophages. PPARγ has been implicated in numerous developmental processes. Disruption of PPARγ gene in mice is lethal during early development: it is required for differentiation of the trophoblast and placental vascularization and homozygous PPARγ-deficient embryos die at day 10 of embryonic development. The PPARγ-null mice that survived to term were deficient in all forms of fat, substantiating the fundamental role for PPARγ in adipogensis, i.e. fat storage. Although PPARγ-null embryonic stem cells were capable to in vitro differentiate into macrophages, it was shown that PPARγ activators enhance monocytic development and PPARγ specific roles have been identified in macrophage lipid metabolism. These were the regulation of oxidized LDL uptake and the transcriptional activation of LXRα̃ Via this latter pathway PPARγ can also contribute to cholesterol efflux by indirectly inducing

ABCA1 transcription. Myeloid specific disruption of PPARγ gene in mice resulted in reduced total plasma and HDL cholesterol levels. In addition, cholesterol efflux was significantly decreased from macrophages elicited by thioglycolate in mutant mice. The lack of the PPARγ gene in macrophages is therefore likely to be pro-atherogenic. Based on this evidence it is not difficult to see that understanding the regulation of PPARγresponsiveness in myeloid cells and macrophages is important and may prove to be therapeutically relevant. We have used myeloid leukemia cell lines blocked at different stages of differentiation and also normal human myeloid cells to study the regulation of PPARγ responsiveness during myeloid maturation. The expression level of PPARγshowed remarkable correlation with the differentiation stage. Previously, retinoids have been implicated in myeloid differentiation on multiple levels but mainly in the granulocytic pathway. Recently, it was reported that retinoids have a role earlier in myelogenesis affecting the common granulocyte/monocyte precursors. This obsevation is consistent with our findings and suggests that it is likely that retinoid action precedes the appearance of PPARγ responsiveness and may contribute to its development during macrophage differentiation.

3.2.Retinoid-PPARγ-LXR crosstalk

We have identified a p450 enzyme CYP27 as a gene commonly induced during monocyte-macrophage transition and as a PPARγ:RXR and RAR:RXR regulated gene in myeloid cells. Promoter analysis revealed complex regulation by retinoid receptors and PPARs via a response element on the promoter of human CYP27 further underscoring the interrelatedness of these pathways. These findings tie retinoid, PPAR and LXR signaling into one regulatory network requiring natural ligands: retinoids and modified fatty acids or prostanoids to activate an entire metabolic pathway and leading to coordinate regulation of lipid/cholesterol uptake, metabolism and efflux. Furthermore, we provided evidence that all components of the described pathways exist in human atherosclerotic lesions.

Applying synthetic ligands to biological systems allows identification of pathways ascribable to the receptors. This proved to be a very fruitful approach to identify biological processes activated by the receptors but it also overwrites the need for the

identification of natural ligands and more importantly sources and regulation of natural ligand production. This approach may also represent superphysiological and ectopic activation of receptors. This is a particular concern in cases of the metabolite receptors (PPARs, LXRs, FXR, PXR) for which only low affinity natural ligands have been identified so far, because activation with low affinity (partial agonist) ligands may lead to a different biological outcome than activating with a synthetic full agonist. Therefore the processes/enzymes leading to endogenous ligand production and their regulation should be also considered when assigning biological functions to receptors. This line of arguments led us to search for mechanisms linking PPARγ:RXR and LXR:RXR signaling pathways. In the case of LXR several low affinity oxysterols have been identified (22(R)-cholesterol, 20(S)-cholesterol, 24-, 25- and 27-hydoxycholesterol as endogenous ligands but none of them has higher affinity than 10 μM. It is important to note that among these oxysterols 27-hydroxycholesterol is present at the highest concentration in the circulation suggesting an *in vivo* relevance in activation of LXR. We identified CYP27 as a nuclear receptor regulated enzyme and capable of producing endogenous ligands for LXR. Moreover, panagonists or RXR activating retinoids can contribute to receptor activation resulting in a robust synergistic response between retinoids and oxysterols. The tying of regulated enzyme expression and heterodimer activation suggests that partial agonists such as 27-hydroxycholesterol can contribute to full activation in the presence of the appropriate retinoid. This adds an additional layer of control to the receptor's activity by the regulation of the production of two endogenous lipid molecules.

CYP27 is an attractive target for regulated transcription because its product is an alternative bile acid synthesis precursor in the liver. Identification of a complex PPAR-RAR regulation and the fact that retinoids present in the serum are regulating the basal expression level of the enzyme in myeloid cells are significant novel aspects of the enzyme's regulation. The product generated, 27-hydroxycholesterol, is a polar compound capable of transversing membranes and therefore provides an alternative cholesterol efflux mechanism. The fact that 27-hydroxycholesterol is an endogenous ligand of LXR has been noted previously. It was even suggested that 27-hydroxycholesterol is a partial agonist of LXR:RXR heterodimers. Our results are in agreement with this assessment, but go further and show that ligand production can be regulated and that, in combination

with retinoids 27-hydroxycholesterol becomes a full agonist on LXR regulated target genes. The fact that retinoids (RAR and RXR selective compounds) and combination of PPAR and RXR selective compounds were able to induce CYP27 expression suggested that there is a cross talk between retinoid and PPAR signaling. It became apparent when the promoter analysis revealed the two enhancers mediating the effects. Further studies identified PRRE-B as the major element and regulator of human CYP27 gene's transcription, whilst PRRE-A might serve as an accessory site required for full activation. It may also be possible that the two elements and two heterodimers are part of a larger complex (i.e. an enhancesome). Traditional promoter/enhancer analysis is not sufficient to dissect such complex relationships. Regulated expression of CYP27 is also of interest because it has a key metabolic function converting cholesterol into a more polar compound, 27-hydroxycholesterol. Consequently it has two major effects on cholesterol efflux. It induces ABC transporter expression and subsequent HDL-dependent efflux via the activation of LXR:RXR. 27-hydroxycholesterol also represents an alternative cholesterol efflux pathway from macrophages that is independent of known transporters including ABCs and HDL and allows converted cholesterol to leave the cells and cleared by the liver as bile acids. It is estimated that under steady state conditions it may represent as much as 10-20 % of total cholesterol efflux as it was shown. Our data suggest that this efflux in macrophages may be regulated by retinoid and PPARγ-mediated induction of CYP27. It is also apparent that besides cholesterol efflux LXR:RXR heterodimers are capable to induce multiple other pathways involved in lipid metabolism such as SREBP1c induction or the induction of phospholipid transport protein and have a more global effect on macrophage lipid homeostasis. As far as the physiological relevance of the pathway is concerned, due to the species specificity (exists in human but not in mouse) of this regulation and the lack of suitable mouse models we had to rely on approaches of gathering data from CTX (CYP27-/-) human fibroblasts and atherosclerotic lesions of humans. The evidence from these approaches clearly demonstrates that CYP27 is required, at least in part, for retinoid and PPARγ ligand induced LXR mediated gene expression and potentially to cholesterol efflux. The striking similarity between gene expression patterns, including retinoid, PPARγ and LXR regulated gene expression as well as high levels of CYP27, of in *in vitro* differentiated

macrophages and in tissue samples of macrophage-rich atherosclerotic lesions also underscores that this regulatory network is likely to have physiological and disease relevance. A recent study suggested that ABCA1 and cholesterol efflux can be regulated directly by retinoids via a RARγ mediated pathway. Our data presented here show that RARα activation has a broader effect on human macrophage cholesterol metabolism as those authors suggested. We suggest that retinoid regulated CYP27 is likely to act as a modulator of robust LXR response. In our view the identified pathway also represents a potential new target for the regulation of macrophage cholesterol efflux and for the management of diseases with increased foam cell formation and cholesterol overload. It also suggests, that retinoids may have more profound effect on lipid metabolism than previously suspected. This notion is further underscored by the observation that RXR agonists have significant atheroprotective effect in apoE-/- mice. Clearly more work needs to be done before regulation of CYP27 can be considered a valid target for pharmacological intervention in these conditions.

3.3. PPARγ - macrophage activation

Macrophages are formed in the tissues from monocytes where they become activated upon various stimuli. Classical activation occurs for example after stimulation of Toll-like receptors and results in the induction of IFNγ, TNFα dependent signal pathway leading to inflammation, induction of anti-proliferative, anti-microbial functions and Th1 dominant immune response. On the contrary, alternative activation is induced by IL-4, IL-13, results primarily in suppressive macrophage functions, elimination of tissue debris, wound healing, Th2 dominant response. Several nuclear receptors have been implicated in various metabolic and immunological functions of the macrophage. Previously, we and others showed that PPARγ, LXRα, RXRα and RARα were highly expressed in macrophages. We sought to characterize these two distinct activation forms of macrophages and find the role for PPARγ in the activated cells. We used human monocytes, mouse peritoneal and bone marrow-derived cells to generate macrophages. We found that PPARγ showed a rapid induction during monocyte-macrophage transition and it was further induced upon alternative activation while was down-regulated upon classical activation. We studied the activity of these receptors by determining target gene

expression levels. Surprisingly, we could show that PPARγ could only be fully activated in alternatively activated and not in classically activated cells. Next, we extended our studies using global gene expression profiling and identified the genes induced in the classically and alternatively activated cells and also the PPARγ-regulated genes. More genes are induced in the alternatively activated cells and the expression levels of the regulated genes are much higher in these cells than in the classically activated ones. These data support the notion that PPARγ is significantly more active in alternatively activated macrophages suggesting roles in this newly defined cell type rather than in classically activated ones. It is very likely that the activation state of macrophages determines the activity of some metabolite activated nuclear receptor pathways.

7. SUMMARY

Nuclear receptors are ligand-activated transcription factors that regulate many aspects of metazoan life. In vivo, a biologically active, functional nuclear receptor requires obviously the protein of the transcription factor, an endogenous ligand that activates it and appropriate conditions in the cell. This latter mainly depends on the presence of elements of co-activator complexes and the permissive, epigenetic status of the regulated DNA. Nuclear receptors have been shown to be important in regulating lipid metabolism in myeloid cells and were also implicated in differentiation processes of the myeloid lineage. Peroxisome Proliferator Activated Receptor γ (PPARγ) appears to be a key component of lipid uptake by inducing the scavenger receptor CD36 that mediates oxidized low-density lipoprotein (oxLDL) uptake to macrophages. Retinoic Acid Receptor (RAR) on the other hand was also shown to play important roles in myeloid cell differentiation.

- We present evidence for a crosstalk between these two nuclear receptor pathways in myeloid cells and show that expression level of PPARγ increases with the degree of monocyte/macrophage commitment. Activation of PPARγ leads to the increased expression of maturation markers (e.g. CD14, CD36). Interestingly, retinoid treatment potentiates PPARγ ability to induce transcription of its target genes.

Cholesterol uptake and efflux are key metabolic processes associated with macrophage physiology and atherosclerosis. PPARγ and Liver X Receptor α (LXRα) have been linked to the regulation of these processes.

- We identified CYP27, a p450 enzyme as a link between retinoid, PPARγ and LXR signaling. We show that the human CYP27 gene is under coupled regulation by retinoids and ligands of PPARs. Induction of the enzyme results in an increased level of 27-hydroxycholesterol that activates LXR and LXR-regulated processes, which leads to an alternative means of cholesterol efflux. Human macrophage rich atherosclerotic lesions have an increased level of retinoid, PPARγ and LXR-regulated gene expression and also enhanced CYP27 levels. Our

findings suggest that nuclear receptor regulated CYP27 expression is a key integrator of RAR-PPARγ-LXR signaling.

- We also analyzed the activation state of macrophages and found that PPARγ is dominantly expressed in alternatively activated macrophages and induces target genes' expression mostly in this specialized cell type.

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