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ENDOCRINE INFLUENCE ON NEUROINFLAMMATION: THE USE OF REPORTER SYSTEMS.

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Abstract

Most of the ageing-associated pathologies are coupled with a strong inflammatory component that accelerates the progress of the physio-pathological functional decline related to ageing. The currently available pharmacological tools for the control of neuroinflammation present several side effects which restrict their application, particularly in chronic disorders. The discovery of the potential anti-inflammatory action exerted by endogenous estrogens and that activation of ERα

results in a significant decrease of inflammation at the cellular level and in models of inflammatory diseases, prompted us to embark in a series of studies aimed at the generation of reporter systems allowing to *i*.) understand the anti-inflammatory action of estrogens at molecular level, *ii*.) evaluate the extent to which the action of this steroid hormone was relevant in models of pathologies characterized by a strong inflammatory component and *iii*.) investigate the efficacy of novel, synthetic estrogens endowed of anti-inflammatory activity. To this aim, we conceived the NFkB-*luc2* reporter mouse, a model characterized by dual reporter genes for fluorescence and bioluminescence imaging under the control of a synthetic DNA able to bind the transcription factor (TF) NFkB, the master regulator of the expression of most of the cytokines responsible for the initial phase of acute inflammation. We here summarize the philosophy that has driven our research in the past years and some of the results obtained so far.

Introduction

Most pathologies associated with ageing and the ageing process itself are characterized by a strong inflammatory component that accelerates the progress of the physiological, or pathology-induced functional decline related to ageing. For instance, the chronic inflammatory activation of neuroglia described in numerous neurodegenerative disorders (Parkinson, Alzheimer and others) is now considered a major cause of the precipitous speed of cell death in the final phases of these diseases (1). Indeed, neurodegeneration would fully involve microglia into clearing the debris associated with neuronal loss and engulfing the misfolded proteins accumulated in the brain parenchyma and therefore distract them from their regular functions (2). Thus, the pathology would limit the number of microglia cells involved in patrolling and reparative functions as well as in the regulation ~~on~~ of neural transmission: this would have severe consequences for brain homeostasis (3-4). The same is true for cardiovascular pathologies such as atherosclerosis where inflammation participates centrally in all stages of this disease, from the initial lesion to the end-stage thrombotic complications (5).

Unfortunately, the pharmacological tools for the control of chronic inflammation available so far are endowed ~~of~~ with numerous side effects which limit their application, particularly in chronic disorders. The discovery that endogenous estrogens have anti-inflammatory potential and that activation of ERα and ERβ results in a significant decrease of inflammation at the cellular level and in models of inflammatory diseases (6-14) led us to initiate a series of studies aimed at the generation of model systems where we could *i*.) understand the anti-inflammatory action of estrogens at the molecular level, *ii*.) evaluate the extent to which the action of this steroid hormone was relevant in models of pathologies characterized by a strong inflammatory component and *iii*.) study the efficacy of novel, synthetic estrogens endowed ~~of~~ with anti-inflammatory activity.

We here summarize the philosophy that has driven our research in the past years and some of the results obtained so far.

Reporter systems

The definition of a reporter gene is: *a gene encoding for an exogenous protein that can be easily detected and quantified*. Several reporter proteins are available; the most used can be classified in two major categories: proteins producing bioluminescence or fluorescence. Bioluminescence reactions involve the oxidation of an organic substrate molecule (called the luciferin) catalyzed by an enzyme called luciferase. There are several luciferases extracted from different organisms (*Photinus pyralis*, *Renilla reniformis, Gaussia*); together with these there are the photoproteins (such as aequorin, and obelin) that are complex proteins where the apoenzyme is associated with the proteic substrate. The generated photons follow a Michaelis-Menten kinetics and, in case of excess of substrate, the light emission is proportional to the amount of enzymatic protein present, thus providing a reliable measure of the activity of the luciferase gene (15). Differently from these bioluminescent compounds, the fluorescent proteins are generally endowed of an intrinsic ability to release photons when excited by a source of light with a very specific wavelength, which depends on the fluorophore structure: e.g. the green fluorescent protein, or GFP, when excited at 400 nm emit fluorescent radiation with a maximum wavelength of 509 nm. The gene encoding GFP has been engineered to improve the brightness of the emission and different color such blue (FP), cyan (CFP), enhanced green (eGFP), and yellow (YFP) (from the shortest to the longest emission wavelength), while the red fluorescent protein mRFP1, emitting at ~600 nm, has been engineered to obtain colors ranging from yellow to red (16). The availability of fluorescent proteins with discriminable emission wavelengths enabled the generation of multi-labelled specimens expressing two or more fluorescent proteins (17).

The detection of bioluminescent processes relies on the administration of specific substrates, while fluorescence imaging requires illumination of fluorescent proteins with a defined wavelength of light ideally near the peak of the fluorophore excitation spectrum. The light emission following the activation of all reporter proteins may be easily measured in living organisms using specific *in vivo* imaging systems featuring highly-sensitive Charge-Coupled Device (CCD) cameras, and specific filters for the detection of emitted fluorescence. The fluorescent signal can be also imaged by *In Vivo* Microscopy (IVM), a technique that requires specific multi-photon microscopes endowed of fluorescence detectors. Bioluminescence imaging is a very sensitive technique that allows rapid detection, and is devoid of problems typical of fluorescence imaging, such as autofluorescence, photobleaching, and biological degradation. On the other hand, for fluorescence imaging the injection of a substrate is not necessary for visualization, a benefit when images at multiple time points over many hours are required. The main limits of all optical imaging modalities are scattering and absorption. Scattering of light at multiple interfaces such as cell and organelle membranes results in highly diffuse emitted light and low spatial resolution. Absorption of light, primarily by hemoglobin, reduces the signal intensity with depth of originating cells in the body. These limitations are particularly evident for fluorescence imaging, and this is partially circumvented using emitters in the red region of the spectrum.

The availability of these proteins, associated with the technology ~~to~~ integrate exogenous DNA into the genome of single cells and whole animals, permitted the creation/conception of model systems where the synthesis of the reporter protein occurred only in the presence of selected stimuli. Thus, these gene products ~~to~~ become surrogate molecules for the quantitative, *spatio*-temporal analysis of a given molecular event in living organisms. So far, one of the best applications of these model

systems is in the investigation of the activity of transcription factors: in this case the reporter of interest, or a combination of reporters can be put under the control of promoters that are target for the given transcription factor (TF): in this way, when the TF is activated it will bind the cognate responsive elements and activate the synthesis of the reporter protein/proteins together with its own targets. The measurement of the extent of bioluminescence/fluorescence produced will be proportional to the state of TF activation and will last as long as the TF will be stimulated, thus providing an insight on the dynamics of the TF mode of action in the different tissues and organs.

A first prototype of reporter mouse was the ERE-Luc mouse where the luciferase promoter had been placed under the control of a synthetic promoter containing multiple copies of the estrogen responsive element. Before integration in the mouse genome, insulator sequences were added to flank and protect the reporter inducible expression from the influence of the surrounding chromatin (18). The reporter mouse obtained proved its usefulness in a series of *in vitro* and *in vivo* studies that enabled us to gain a major insight into the unsuspected actions of estrogens receptors in non-reproductive organs (19), and paved the way for the generation of new, advanced and more performant reporter mice. One of the advantages of these new reporter mice is the presence of the reporter genes under transcriptional control of responsive elements of biological processes of interest, while, traditionally the reporter systems were generated by knock-in insertion of a reporter gene downstream of the selected promoter. The limit of this application was that it should disrupt the functionality of target genes in one allele with unknown consequences on the phenotype and most likely it limited the use of these mice to heterozygous. Moreover, this method limited the analysis at one specific promoter by keeping out the general biological processes. A second advantage is the generation of reporter mice by homologous recombination and not random integration, the main advantages of targeted transgenesis is that the mice generated carrying a single copy of the transgene integrated into a predetermined site of the genome that are proven to allow strong and predictable expression of inserted transgenes (20). The choice of a good performant permissive genomic locus is fundamental to generate a transgenic reporter mouse but also the use of insulator sequences (18) that can function as directional blocking elements either by interfering with promoter-enhancer interactions when positioned in the intervening sequence or by reducing position effects imparted on transgenes when flanking the integrated transcription units.

The generation of a reporter system to study the evolution of the inflammatory process in living cells and animals

A brilliant example is provided by the NFκB-*luc2-IRES-Egfp* reporter system, that we conceived to investigate the dynamics of estrogen action on the inflammatory processes. The system is composed of a dual reporter containing the genes encoding the luciferase and the green fluorescent protein *(luc2-IRES-Egfp)*; both genes had been engineered to obtain enhanced fluorescence and bioluminescence (21, 22). The two genes were separated by an internal ribosome entry site (IRES), a DNA element encoding a RNA that allows for translation initiation in an end-independent manner, as part of the greater process of protein synthesis (23). For the promoter we utilized a DNA sequence able to bind the TF NFκB, the master regulator of the expression of most of the cytokines responsible for the initial phase of acute inflammation (24, 25, 26). Such promoter was completely synthetic and was the resultant of a thorough analysis of a panel of genes involved in innate immunity which led us to identify two major groups of NFκB consensus sequences. These sequences were selected and

used for the generation of an array of promoters containing multiple copies of such sequences in different arrangements (Fig. 1). Studies in transient as well as in stably transfected cells using the generated NFκB reporters, enabled us to identify a reporter construct activated by several pro-inflammatory stimuli, but not by the anti-inflammatory cytokine interleukin-4 (IL4) (27). Using the isolated reporter, we have been able to study for the first time the effect of estrogen administration in monocytes stimulated by a bacterial toxin (lipopolysaccharide, LPS) to deploy an inflammatory reaction (Fig. 2). Contrary to what was previously believed (11), we found that the presence of estrogens did not completely prevent NFκB translocation into the nucleus; however, in the cells in which LPS successfully induced NFκB transcriptional activity, the presence of estrogens accelerated, time-wise, the inflammatory response. After the stimulation, the cells treated with estrogens rapidly acquired a phenotype associated with the activation of repair mechanisms and characterized by the expression of trophic factors such as *IL10* and *TGFβ*. The study therefore provided a mechanistic explanation for the beneficial effects of estrogens in wound healing, stroke or selected neurological disorders where stimulated macrophages and microglia acquire a status of permanent activation causing damages and death of the cells surrounding the site of inflammation. In addition, this model system revealed that in macrophages there is a complex interplay between the membrane estrogen receptor GPR30 and the intracellular ERα that facilitates ERα-dependent activation of the genes relevant for the acquisition of the reparative phenotype (e.g. *Socs3* and *IL-10*). The interplay between the two receptor systems has to be taken into account for the pharmacological exploitation of estrogen anti-inflammatory activity.

The study of NFkB activity in whole, living organisms

The promoter cassette developed was further modified for the generation of the reporter mouse and enable to its integration by homologous recombination into a permissive locus recently described by our group (28). Considering the large amount of cell expressing the NFκB transcription factor, we opted for a methodology allowing the tissue-specific expression of the reporter: to this aim, we integrated a stop sequence flanked by cre-Lox sites in between the promoter and the reporter cassette, in addition we added insulator sequences (29, European patent No. EP 1298988B1; US patent No. 7,943,81) (Matrix attachment Regions, MAR) to decrease the probabilities of interference of the expression of the NFκB -driven promoter by sequences surrounding the genomic integration sites. As reporters, we selected a bicistronic system with the *luc2* gene for *in vivo* bioluminescence measurement and a gene encoding a protein ideal for *ex vivo* studies at single cell level (tdTomato, a monomeric red fluorescent protein derived from the Discosoma sp. red fluorescent protein) (17). As expected, the mice carrying the entire transgene expressed a negligible amount of luciferase (Fig. 1c). After breeding with the B6.C-Tg(CMV-cre)1Cgn/J mouse expressing the Cre recombinase in germ cells, the resulting generations of mice showed a generalized, basal, expression of the reporter (Fig.1c). We next tested whether this basal bioluminescence could be increased by a treatment with a pro-inflammatory molecule such as LPS. Fig.2a shows a strict correlation between the dose of LPS administered and whole body bioluminescence emission (BLI). When we analyzed the response of selected body areas representing different organs we noticed that the luciferase expression was dose-dependent in all body areas with the exception of the uterus where the expression of the reporter reached maximal levels after an intermediate dose of LPS. The fact that not all tissues expressed the same amount of luciferase was ascribed to differences in the presence of cells for the innate immunity and in the

distribution of i.p. injected LPS (e.g. brain). Indeed, these results were consistent with subsequent analyses on the expression of the endogenous target genes (30).

To finally demonstrate that estrogens are able to accelerate the inflammatory response by speeding up the activity of NFκB and rapidly decrease it, we measured luciferase expression in time in mice treated with LPS alone or LPS + 17β-estradiol (E_2). To this aim, male NFκB mice of 4 month of age were treated with E_2 (10μg/kg) 16 h prior to administration of LPS. The hormonal treatment was repeated at 0, 9, 32, 48, 55 and 72 h to maintain adequate concentrations of the hormone in plasma and BLI was measured at 0, 3, 6, 9, 32, 48, 55 and 72 h by *in vivo* imaging. Fig.3 shows that, indeed, the increase in luciferase expression was hastened in the animals treated with the hormone with a peak of activity at 3 h; at 72 h the extent of bioluminescence was lower in these same animals. Thus, even the *in vivo* analysis of LPS activity indicated that estrogens can limit in time the inflammatory response.

Conclusions

The NFκB-*luc2* reporter mouse is a novel tool for the study of inflammation and neuroinflammatory processes. The construct generated has been integrated in a known *locus* allowing for the generalized and NFκB regulated expression of the dual reporter *luc2*-TdTomato. Luciferase offers the advantage of whole body imaging or the study of luciferase expression *ex vivo* in tissue extracts or in brain slices (31), while TdTomato enables the study of NFκB activation at cellular level in living cells or in tissue slices.

The validity of the system is that the minimal synthetic promoter enables the study of the state of activation of the TF. Indeed, each endogenous NFκB target has a tissue-dependent sensitivity to inflammatory stimuli because it is under the control of complex promoters controlled by a multiplicity of transcription factors that may act *spatio*-temporally in a different way. Thus, considering the expression of endogenous targets, it is difficult to obtain a conclusive indication of the extent of transcriptional activation of NFκB in each tissue.

Thus, this experiment further underlined the usefulness of the use of a surrogate reporter of TF activity as less susceptible than complex promoters to unpredictable influences able to enhance/suppress the activity of the TF on that promoter, but not on other targets.

The breeding of the NFκB reporter mouse with animal models of neurodegeneration will facilitate the study of the impact of neuroinflammation on the onset and evolution of these pathologies. In addition, the accessibility to this mouse will facilitate the screening and study of novel anti-inflammatory compounds.

Moreover, the use of reporter mice allows reaching all these goals with a significant reduction of the number of animals to be used and of their distress, in the full compliance of the 3R regulations.

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Figures legends

Figure 1.

Generation of a reporter system for the study of NFκB activity in cells and reporter mice

a) generation of the reporter for cell transfection;

b) testing of the validity of the synthetic responsive element in stably transfected cells;

c) modification of the reporter cassette with the substitution of EGFP with TdTomato, integration of the stop cassette flankeb by loxP sequences for the tissue specific expression.

Figure 2.

a) *In vivo* imaging of the NFκB reporter mouse after injection of vehicle or LPS at increasing dosage;

b) semiquantitative analysis of photon emission from the different region of interest (ROI).

Figure 3.

Estrogen accelerates the resolution of LPS-induced inflammatory response in NFκB reporter mice.