Technology and Development Team for BioSignal Program

Subteam for BioSignal Integration

Subteam Leader, Takahiro DOI

Goal Lives always receive stimulation from outside and maintain themselves in response to such stimulation through the complex network of signal transduction. Analysis of this mechanism is the way to elucidate bio response mechanism and to characterize bioresource materials. The main goal of our subteam is to develop new techniques for elucidation of signal transduction mechanism on bio response through the analysis of gene and protein expression using microchip array technique and database at each step including genes, cells, tissues and whole bodies. And the other goal is elucidation of the effect toward lives by abnormal cytokine networks and the mechanisms of diseases including cancers through analysis of signal transduction pathways of cytokines. Furthermore it's the another goal of ours is to develop the model mice for human diseases with the reconstruction of abnormal networks in vivo in the base of the bioinformation stored in BRC for the more detailed study and development of the new treatment for those diseases. We focus on characterization of NF-*k*B and Tumor necrosis factor (TNF) for analysis of signal transduction mechanisms in lives.

Activities

1. Analysis of the essential roles of NF-kB in tumorigenesis:
2. Analysis of the essential roles of NF-kB in apoptosis pathway
3. Analysis of the essential roles of NF-kB in innate immunity
4. Analysis of the essential roles of NF-kB in development
5. Development of the new technology for the expression profiling

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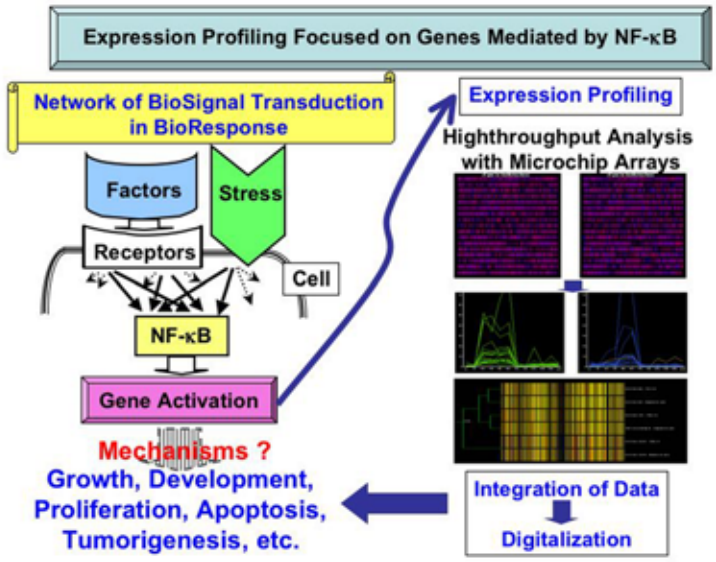

Figure 1. Analysis of maechanism of signal transduction network in lives.

Specific aim

I. Analysis of the essential roles of NF-kB in tumorigenesis:

There're a lot of factors which are related with the steps for tumorigenesis. We have been focused on characterization of NF-kB among those factors for analysis of the mechanism of tumorigenesis.

(1) Mechanism of tumor suppression by NF-kB:

It has been demonstrated that NF-kB might be related with tumorigenesis rather than tumor suppression. However we detected tumor susceptibility in TNF/RelA double knockout mice. And we also demonstrated that NF-kB/RelA deficient cells derived embryos turned to be transformed with high frequency compared to the cells derived from normal mouse embryos. The growth ratio is quite rapid both in vivo and in vitro. And those cells formed tumor mass in mice bodies after subcutaneous injection. High-throughput analysis of expression profiling using those cells demonstrated that there's significantly high expression of genes involving oncogenes, cell cycle-related genes, anti-apoptotic genes. Therefore it is demonstrated that NF-kB might have the tumor-suppressing effects.

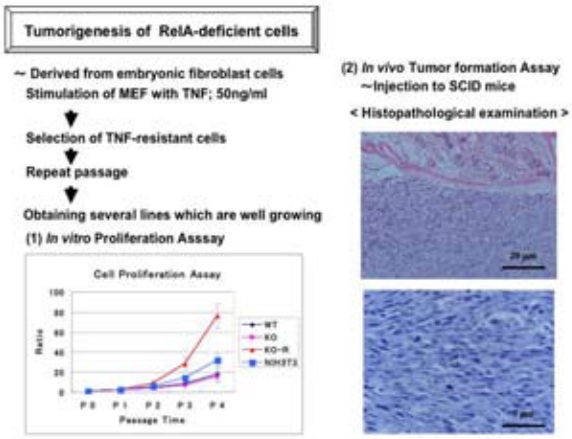

Figure 2. Analysis of the mechanism for tumor suppression by NF-kB

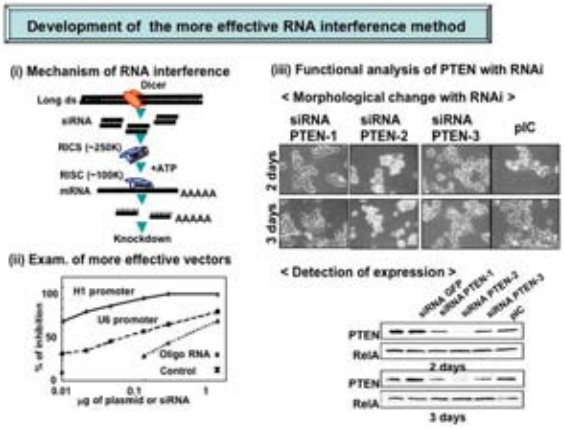

Figure 3. Functional analysis of suppressor oncogene PTEN with RNA interference

(2) Analysis of the function of suppressor oncogene, PTEN using RNA interference method:

PTEN is one of tumor suppressor genes closely related to tumorigenesis. It is very difficult to characterilize that function, because PTEN knockout mice are lethal. We generated the cells that have extremely decreased PTEN activity with RNA interference method for analysis of PTEN function. Those cells lost the adhesion activity. We detected that phenotype was due to dysregulated cytoskeleton with decrease of FAK and actin fibers. And we also demonstrated that phenotype was not caused by non-specific effects of RNA interference for PTEN.

II. Analysis of the essential roles of NF-kB in apoptosis pathway

(1) Analysis of apoptosis pathway by TNF

Previously we generated NF-kB/RelA knockout mice and detected that those mice were embryonic lethal due to TNF cytotoxicity. That phenotype demonstrated that NF-kB/RelA had anti-apoptotic activity against TNF cytotoxicity. We tried to elucidate anti-apoptotic factors that are transcriptionally activated by RelA. With analysis using fibroblast cells derived from RelA-deficient embryos, it was detected that Caspase-dependent pathway was activated, while mitochondria-dependent pathway was not activated. For identification of anti-apoptotic genes directly mediated by RelA, we did high-throughput analysis of gene expression profiling with chip arrays using RelA-deficient cells and normal cells. Among a lot of genes, Bcl-2 family genes, typical antiapoptotic genes, were not detected, but IAP (Inhibitor of apoptosis protein) family genes were detected. However RelA-deficient cells were not rescued from TNF cytotoxicity by introduction of those IAP genes. Meanwhile we detected decrease of c-FLIP, an inhibitor of Caspase-8, in RelA-deficient cells with Western blotting. And introduction of c-FLIP rescued RelA-deficient cells from TNF cytotoxicity. Therefore it was speculated that RelA might regulate c-FLIP expression indirectly. We are now analyzing how RelA regulates c-FLIP expression. We analyzed macrophages lacking RelA also. RelA-deficient macrophages are sensitive to TNF, although those cells have c-Rel protein, another NF-kB family member. This evidence demonstrated that only RelA

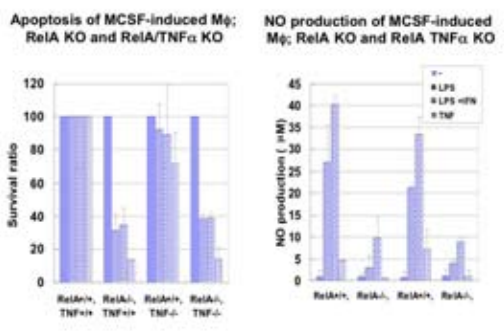

Figure 4. Analysis of RelA deficient macrophages

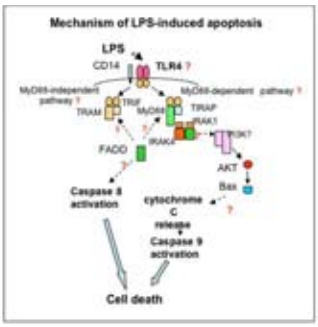

Figure 5. Analysis of Caspase activity

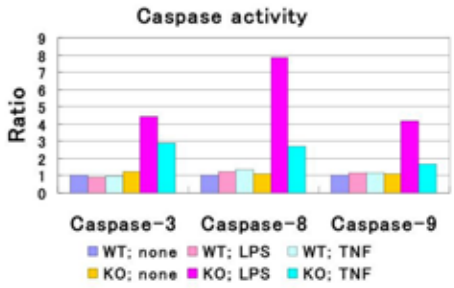

Figure 6. Mechanism of LPS-induced apoptosis

might play a critical role on anti-apoptosis against TNF cytotoxicity in specific tissues.

(2) Lipopolysaccharide (LPS)-induced apoptosis and NF-kB

RelA-deficient macrophages are apoptotic by LPS, although normal macrophages are activated by LPS. We detected that Caspase-dependent pathway and mitochondria-dependent pathway both were activated in apoptosis of RelA-deficient macrophages by LPS. We confirmed that apoptosis of RelA-deficient macrophages by LPS were not due to TNF induced LPS, but directly due to LPS, with demonstrating that TNF/RelA-deficient macrophages were also apoptotic with LPS. And we demonstrated that there might be the mechanism that activated Caspases just at downstream of receptor for LPS, e.g. TLR4, which might be similar to TNF receptor 1.

Any types of c-Rel-deficient cells are not apoptotic neither by TNF nor LPS. This demonstrates that only RelA plays a critical role on anti-apoptosis by TNF/LPS among NF-kB family members.

III. Analysis of the essential roles of NF-kB in innate immunity

It has been thought that NF-kB might play the critical roles on innate immunity, although there is no obvious evidence demonstrated so far. We obtained some evidence for that hypothesis.

RelA deficient mice that we once generated are embryonic lethal. We transplanted fetal livers from RelA-deficient embryos to Ly5.1 mice for analysis of hematopoiesis of RelA-deficient stem cells. Surprisingly matured blood cells and lymphocytes appeared in mice transplanted with RelA-deficient fetal livers. And those lymphocytes had a little decreased reactivity to several stimuli that promoted proliferation for normal cells. Next we challenged Leishmania major to those transplanted mice for analysis of the response of RelA-deficient immune cells against infection. Mice carrying RelA-deficient cells had severe swelling after challenge of Leishmania major and died, like Balb/c mice that had no TH1 lymphocytes that were

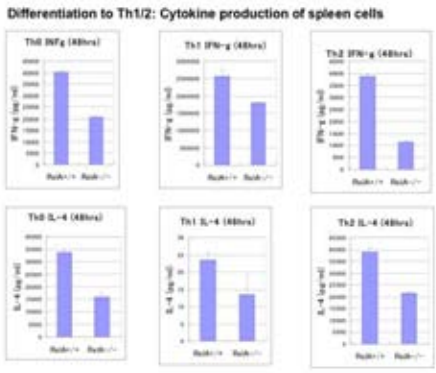

Figure 7. Analysis of potentiality for TH-1/2 induction

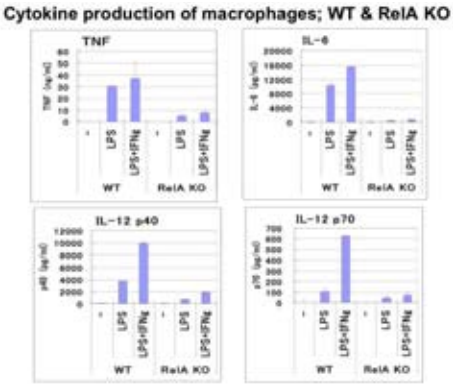

Figure 8. Analysis of cytokine production activity

induced with infection. That evidence demonstrated that RelA-deficient lymphocytes had no potentiality for development to TH1 cells even after infection. More analysis showed that RelA-deficient macrophages had severe defect of producing activity of inflammatory cytokines; TNF, IL-12 or IL-6. These demonstrated that RelA might play the critical roles on innate immunity, especially on host defense against infection. However mice only lacking c-Rel had no symptoms for the same challenge.

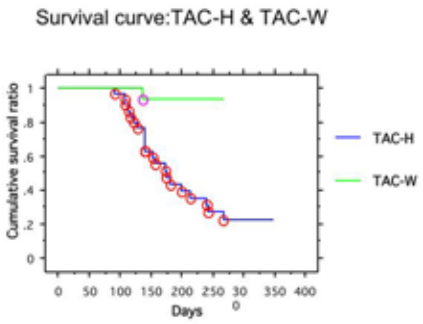

Figure 9. Analysis of viability of c-Rel/RelA eficient mice

We generated TNF-/-RelA-/ mice and TNF-/-c-Rel-/-RelA+/- mice for further analysis. Both of those mice died several weeks after birth due to infection. Analysis of causal death revealed that those mice died due to Pasteurella pneumotica and Staphylococcus aureus both of which had quite weak pathogenic. That demonstrated that both of mice were much mode severe immunodeficient mice than SCID mice or nude mice. More analysis revealed that TNF-/-RelA-/- mice had neither activity producing nitric oxide which killed bacterias nor potentiality for inducing TH1 lymphocytes. TNF-/-c-Rel-/-RelA+/- mice had defect on producing neutrophil proteinases that killed bacterias and severely decreased activity of cytokine production.

These demonstrated that NF-kB might play the critical roles in innate immunity coordinately with TNF.

IV. Analysis of the essential roles of NF-kB in development

We have been analyzing how NF-kB functions in development. So far there has been no evidence that RelA or c-Rel has any potentiality for development, although dorsal, Drosophila homolog of NF-kB, plays a critical role on determination of dorsal-ventral axis in embryonic stages. However in this case we show that NF-kB plays a critical role on organogenesis. We generated TNF-/-c-Rel-/-RelA-/- mice for rescue of c-Rel-/-RelA-/- mice from TNF cytotoxicity. TNF-/-c-Rel-/-RelA-/- mice were born, while TNF+/+c-Rel-/-RelA-/- mice died at 13.5 d.p.c. However those mice died several hours after birth. Pathological findings demonstrated that lungs of newborn mice were not dilatated. The cause was that those mice had no pulmonary surfactants in lung epithelial cells. That demonstrated that NF-kB might play a critical role on lung organogenesis in neonatal stage.

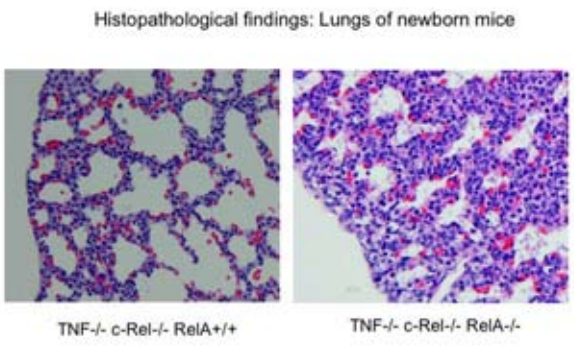

Figure 10. Histopathological analysis of c-Rel/RelA deficient mice

For analysis of hematopoiesis activity of cells lacking both of c-Rel and RelA, we transplanted fetal livers from embryos lacking c-Rel and RelA. Analysis of spleen, bone marrow and thymus in those transplanted mice revealed that matured lymphocytes appeared. These demonstrated that neither RelA nor c-Rel is essential for lymphocyte development, although both of them were identified as the critical factors originally.

V. Development of the new technology for the expression profiling

For further analysis of gene network, we are developing the new system for analysis of expression profiling of functional RNA. So far we are doing high-throughput analysis of antisense RNA expression with chip arrays.

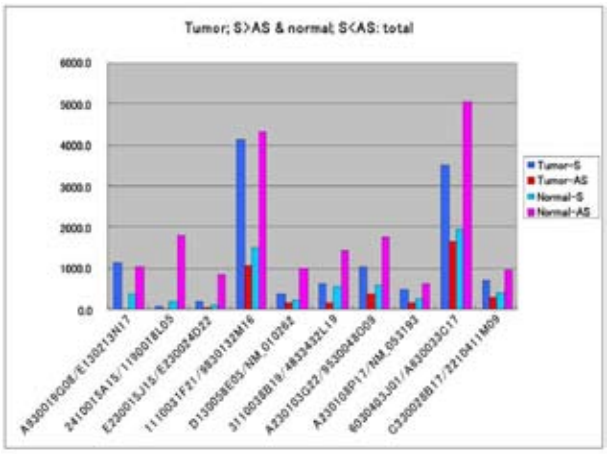

Figure 11. Expression profiling analysis of an t isense RNA

The tumors that we used were mammary tumors that develop spontaneously with high frequency in GRS/A mice. Significant difference between tumors and normal tissues was detected about several antisense RNAs. And almost of those were non-coding RNAs. So far our analysis of antisense RNA using tumors is the first in the world. We are now analyzing the correlation between antisense RNA expression and tumorigenesis for clinical application of this technique in the future.

Publications Original Papers (*Peer reviewed Journal)

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Takahiro Doi, Hidenori Kiyosawa, atsushi Yoshiki, Noriko Hiraiwa Expression profiling analysis of antisense RNA in mouse tumors The 27th MBSJ Annual Meeting, Kobe, December, 2004

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Setsuko Mise, Takahiro Doi NF-κB/RelA subunit plays a critical role in macrophage apoptosis. The 35th Annual Meeting of the Japanese Society for Immunology, Yokohama, December, 2005