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Review

Rescuing Developing Thymocytes from Death by Neglect

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The major function of the thymus is to eliminate developing thymocytes that are potentially useless or autoreactive, and select only those that bear functional T cell antigen receptors (TCRs) through fastidious screening. It is believed that glucocorticoids (GCs) are at least in part responsible for cell death during death by neglect. In this review, we will mainly cover the topic of the GC-induced apoptosis of developing thymocytes. We will also discuss how thymocytes that are fated to die by GCs can be rescued from GC-induced apoptosis in response to a variety of signals with antagonizing properties for GC receptor (GR) signaling. Currently, a lot of evidence supports the notion that the decision is made as a result of the integration of the multiple signal transduction networks that are triggered by GR, TCR, and Notch. A few candidate molecules at the converging point of these multiple signaling pathyways will be discussed. We will particularly describe the role of the SRG3 protein as a potent modulator of GC-induced apoptosis in the crosstalk.

Keywords: Apoptosis, Glucocorticoids, SRG3 protein

Introduction

The thymus plays a pivotal role in the differentiation and selection of functional T cells during T cell ontogeny. A major function of the thymus is to eliminate developing thymocytes that are potentially useless or autoreactive, and select only those that bear functional T cell antigen receptors (TCRs) through fastidious screening (Sebzda *et al.*, 1999). The key event in thymocyte development is initiated at the TCR/CD3 complex. Cells that are capable of transducing TCR-mediated signals undergo rigorous selection, but the vast majority of the incapable cells are eventually eliminated by apoptosis. The latter event of cell death is named 'death by neglect'. The molecular mechanism of death by neglect is unclear, but the common view is that the cells die due to lack of 'survival signals' that are delivered in subsequent to the TCR-ligand interactions. $CD4^+CD8^+$ DP thymocytes (their TCRs are incapable of recognizing self-peptide/self-MHC complexes, due to the nonproductive rearrangement of their germline TCR-α and -β genes or expression of TCRs with subthreshold avidity) are fated to undergo apoptotic death (Alam *et al.*, 1996; Alam *et al.*, 1999). Moreover, in addition to the lack of TCR-mediated signaling, other signaling cascades may be implicated in the death of neglected thymocytes (Amsen and Kruisbeek, 1998). There are numerous molecules on the surface of thymocytes, such as co-receptors, cytokine receptors, co-stimulatory molecules, and adhesion molecules, which can transmit intracellular signals with the potential to modulate TCR-mediated signals. In addition to these surface molecules, intracellular signaling mediators seem to play a role in this death process. Among them, steroids that are produced in the thymus or transferred in an endocrine manner have long been known to affect thymocyte development. A number of studies on the effect of steroids on the thymus (for example, the early observation of an increase in thymocyte number by adrenalectomy that was first reported in 1924) has led immunologists to believe that circulating or endogenous steroids, most likely glucocorticoids (GCs), are responsible, at least in part, for cell death during death by neglect (Ashwell *et al.*, 2000). In this review, we will mainly cover the topic of GC-induced apoptosis of developing thymocytes. We will also discuss how thymocytes that are fated to die by GCs can be rescued from GC-induced apoptosis in response to a variety of signals with antagonizing properties for GC receptor (GR) signaling. Although extensive studies on the multiple signaling events that are involved in making the life or death decisions of DP thymocytes are still underway, the exact molecular mechanism of this apoptotic event still needs to be clarified. Currently, a lot of evidence supports the notion that the decision is made as a result of the integration of the multiple signal transduction networks that is triggered by GR, TCR, and Notch. A few candidate molecules at the converging point of these multiple signaling will be also discussed. Particularly the role of the SRG3 protein as a

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potent modulator of GC-induced apoptosis in the crosstalk will be described.

GC-induced apoptosis

By the early twentieth century, it was already known that stress and the drug-induced involution of the thymus is prevented by adrenalectomy (Jaffe, 1924a, b). Subsequently it was found that the administration of the adrenocorticotrophic hormone (ACTH) to mice caused a marked reduction in the thymus and lymph node mass (Dougherty and White, 1943; Selye, 1936). Today we understand that the mass reduction in the thymus is a result of the apoptosis of DP thymocytes, and that this cell death is caused by the ACTH-induced adrenal steroid, glucocorticoid (GC) (reviewed in Ashwell *et al.*, 2000).

Due to its lipophilic property, GCs that are free of a protein carrier passively diffuse across the plasma membrane in the cell where they encounter GR. GR is composed of three functional domains. They are as follows: a constitutive N-terminal activation domain, a central DNA-binding domain, and a C-terminal ligand-binding domain, which also contains residues that are required for dimerization and hormone-dependent gene transactivation (Beato *et al.*, 1995). Upon activation by GCs, GR homodimerizes and translocates to the nucleus where it binds to specific response elements in DNA to either activate or repress transcription of target genes (Beato, 1991).

Since GC-induced apoptosis was prevented in the presence of the transcription or translation inhibitors (such as actinomycin D or cycloheximide, respectively), GC-induced apoptosis has been shown to require protein synthesis (Wyllie *et al.*, 1984). Along with the early observations that showed the essential role of GR in GC-induced apoptosis, this result implies that GCs trigger the induction of so-called 'death genes' or 'lysis genes' via steroid-activated GR to induce apoptosis. Although several GC-induced genes have been discovered, none of them have been shown as uniquely responsible for GC-dependent apoptosis. On the other hand, the role of GR as a repressor suggested the existence of 'survival genes' that are transcriptionally repressed by active GR (Helmberg *et al.*, 1995). Bcl-2, Bcl-X_L, and IAPs (inhibitors of apoptosis) are among the few candidate genes, but the exact mechanism of their transcriptional regulation remains to be seen in the context of their individual gene promoters (Sentman *et al.*, 1991; Grillot *et al.*, 1995; Duckett *et al.*, 1998). However, it is unlikely that activated GRs transcriptionally induce or repress the death- or survival-specific genes, respectively, which exert direct lethal effects on T cells. Rather, it seems that GCs lead to the activation of the apoptotic enzyme, which is constitutively expressed as a proenzyme by altering the profiles of the genes that are expressed (Thompson, 1999).

At present, it is clear that the GR-mediated transcriptional regulation of a still unidentified gene(s) is important for the induction of apoptosis. The GR can modify gene transcription as a homodimer by directly binding to the palindromic glucocorticoid response elements (GREs). Furthermore, it was suggested that GR could also regulate the transcription of various genes whose regulatory regions have no DNA-binding sites for GR (Beato, 1991). In this case, GR, as an activated monomer, associates with other transcription factors, such as AP1, NF-κB, CREB, or STATs, via direct protein-protein interactions, which may yield simple sequestration of these transcription factors into inactive complexes (Jonat *et al.*, 1990; Yang-Yen *et al.*, 1990; Imai *et al.*, 1993; Ray and Prefontaine, 1994; Stocklin *et al.*, 1996; Zhang *et al.*, 1997). Furthermore, GR and other transcription factors were shown to compete for limiting co-activators ('squelching') (Adcock *et al.*, 1998; Kino *et al.*, 1999; McKay and Cidlowski, 2000). For a GR-mediated repression of NF-κB activity, additional mechanisms were also demonstrated - the cytosolic retention of NF-κB dimers in an inactive form by GC-induced IκB, or the inhibition of serine-2 phosphorylation of the RNA polymerase II carboxyl-terminal domain upon binding of GR to p65 RelA subunit of NF-κB (Auphan *et al.*, 1995; Scheinman *et al.*, 1995; Ito *et al.*, 2000).

Reichardt *et al.* utilized an ingenious transgenic-mice system to dissect the mechanism of GR action (Reichardt *et al.*, 1998). They have knocked-in a point mutation into the GR gene, which renders a dimerization-defective GR (GR^{dim}) that no longer binds cooperatively to GREs. The GR^{dim} was defective in DNA-binding-dependent gene activation, but interference with AP1 activity via protein-protein interaction remained intact. Since GR was reported to act as a transcriptional regulator by directly binding to GRE or interacting with other transcription factors, respectively, this system was useful for assessing the contribution of the DNA-binding of GR, compared with the transcriptional interference by GR to the GC-induced apoptosis. Interestingly, DP thymocytes from $GR^{dim/dim}$ mice were fully resistant to GC-induced apoptosis while the cellularity and CD8/CD4 ratio were not significantly changed. Indirect mechanisms (e.g. by interference with or enhancement of the activities of other transcription factors) appear to be unnecessary for GR-mediated apoptosis. Rather, it is more likely that direct gene regulation by GR is necessary for GCs in order to induce apoptosis. However, as stated earlier, such GR-induced death genes still need to be identified.

In addition to its role as a transcription factor itself or an indirect transcriptional regulator, GR can regulate gene expression by remodeling the chromatin structure. The GR is reported to interact with chromatin-modifying complexes such as ATP-dependent switch/sucrose non-fermentable (SWI/SNF), histone acetyltransferase (HAT), and histone deacetylase (HDAC) complexes (reviewed in Collingwood *et al.*, 1999). Östlund Farrants *et al.* reported that the association of GR to GRE stimulates the nucleosome disruption by the biochemically purified and reconstituted mammalian SWI/SNF complex (Östlund Farrants *et al.*, 1997). Fryer and

Archer found that the recruitment of the mammalian SWI/SNF complex to the GR *in vivo* was dependent on the presence of GCs in the breast cancer cell line T47D (Fryer and Archer, 1998). A recent report from Wallberg *et al.* demonstrated that the major transactivation domain τ1, located in the N terminus of GR, directly interacts with the purified yeast SWI/SNF complex (Wallberg *et al.*, 2000). Although the exact biochemical components of the murine SWI/SNF complex still need to be elucidated, GR in the murine thymus was shown to form a complex with SRG3, the mouse homolog of human BAF155 (Han *et al.*, 2001). Furthermore, the GRE-dependent reporter activity was reduced in the S49.1 thymoma cell line that expressed the antisense SRG3. In an inhibitor study of Plesko *et al.* using the HDAC inhibitor, sodium butyrate, HDAC activity was required for the GRE-dependent tyrosine aminotransferase gene expression in hepatoma cells (Plesko *et al.*, 1983). Recent studies of Ito *et al.* have shown that GR represses NF-κB-mediated HAT activity by a combination of the direct inhibition of CBP-associated HAT activity and the recruitment of HDAC2 to the NF-κB activation complex, p65-HAT complex (Ito *et al.*, 2001). However, the exact role(s) of these chromatin-modifying complexes in the thymocyte differentiation is still unknown.

The molecular pathways beyond receptor transactivation that lead to GC-induced apoptosis are not fully understood. One major characteristic of GC-induced apoptosis is the dependence on gene expression (Mann *et al.*, 2000). Multiple studies with transcription and translation inhibitors revealed the requirement of de novo protein synthesis in various biochemical events. They are as follows: the activation of calcium-dependent endonuclease (Cohen and Duke, 1984), the elevation of the cytosolic calcium concentration (McConkey *et al.*, 1989), the activation and selective translocation to the particulate fraction of protein kinase C epsilon (PKCε) (Iwata *et al.*, 1994), the production of reactive oxygen species and the depletion of reduced glutathione (Fernandez *et al.*, 1995), and the loss of membrane potential (Mann and Cidlowski, 2001). Recently, a novel low molecular weight nuclease, NUC18, was identified as the calcium-dependent endonuclease (Cidlowski *et al.*, 1996). Currently, it is believed that gene regulation is an early step in the GC-induced pathway after the GC-GR interaction, and that gene regulation serves as a molecular switch that controls the progression of GC-induced apoptosis. GC-induced apoptosis is mediated via the mitochondrial pathway that is distinct from the TCR-induced apoptosis. GC-treatment disrupts the outer membrane of mitochondria, releasing pro-apoptotic molecules like cytochrome c and AIF (apoptosis inducing factor). These molecules finally activate caspase-3 (also known as CPP32), effector of apoptosis, through the actions of Apaf1 and caspase-9. Bcl-2, Bcl-X_L, and IAPs are well known as powerful modulators to block the lethal effect that is induced by GCs (Sentman, *et al.*, 1991; Grillot, *et al.*, 1995; Hakem *et al.*, 1998; Kuida *et al.*, 1998; Yoshida *et al.*, 1998). Finally, a few signaling molecules are reported to be modulated upon GC treatment. They are as follows: the generation of diacylglycerol (DAG) through protein kinase C (PKC) and G-protein-dependent phosphatidylinositol-specific phospholipase C (PI-PLC), the activation of acidic sphingomyelinase (aSMase), and the generation of ceramide (Cifone *et al.*, 1999). These observations indicate that GC-induced apoptosis occurs through a complex signaling pathway(s) that requires the sequential activation of different biochemical events.

The effect of modified GR signaling on thymocyte development

There is a substantial body of evidence that suggests that the strength of TCR signaling can be regulated by GCs. Many researchers reported the alteration of thymocyte development in mice with modified GR signaling. They believe this alteration may be caused by the modulation of TCR signaling by GCs in the thymus. Recently, by conducting various biochemical analyses (including co-immunoprecipitation and kinase assays after subcellular fractionation), Laethem *et al.* demonstrated that the membrane-proximal signaling that is triggered by TCR is attenuated by GCs in the 3B4.15 murine hybridoma cell line and primary thymocytes, leading to the conversion of agonist signals into partial agonist-like signals (Baus *et al.*, 1996; Van Laethem *et al.*, 2001). They showed that GCs exerts its inhibitory effect on TCR proximal signaling by interfering with the early phosphorylation events induced by TCR engagement. Inappropriate phosphorylation events were caused by the disruption of membrane raft by GCs. This led to both the dispersion of signaling molecules (such as $p56^{lck}$ and $p59^{fyn}$) in the soluble membrane fraction and the hypophosphorylation of CD3ε and ζITAMs (p23) due to ineffectively clustering them with the relevant receptor tyrosine kinases (RTKs). This sequestration of RTKs in ineffective complexes, or the inhibition of juxtaposition of RTKs with their substrates, eventually resulted in the hypophosphorylation of ZAP-70, LAT, and PLCγ1. It is interesting that they provide a plausible explanation on the role of GCs in both thymus and periphery. By attenuating the TCR signaling directly, thymic GCs may play a role in shifting the optimal window of positive selection toward higher TCR avidities for self-peptide/self-MHC complexes in order to establish sufficient reactivity to self-MHC in the periphery. Similarly, peripheral GCs may also reduce the strength of TCR signaling in naïve T cells, and drive Th2 cell differentiation favorably in response to antigen. However, it is necessary to observe thymic development in the microenvironment of various dosages of GR or GCs, in order to determine if GCs affect T-cell development. In recent years, several groups reported the effects of attenuated GR signaling on thymocyte development.

Vacchio *et al.* analyzed the effect of metyrapone, an inhibitor of GC biosynthesis, on thymic selection (Vacchio *et al.*, 1994; Vacchio and Ashwell, 1997; Vacchio *et al.*, 1999). When fetal thymic organ culture (FTOC) was performed with

thymi from RAG2$^{-/-}$H-YTCR^{+} female mice (H-2^{b}), a blockade of the GC production by metyrapone led to the substantial apoptosis of DP thymocytes that would be positively selected in the absence of metyrapone. However, metyrapone did not affect H-YTCR^{+} thymocytes in the H-2^{d} non-selecting background (Vacchio and Ashwell, 1997). These results imply that GCs in the thymus lead to the apoptosis of DP thymocytes that normally undergo positive selection by inhibiting the TCR signaling. However, because metyrapone blocks the action of P450c11 that converts dexoycorticosterone (DOC) into corticosterone, its treatment may lead to a build-up of DOC, which is a potent mineralocorticoid with partial GR agonist activity (Brown and Strott, 1971; Lechner *et al.*, 2000). Furthermore, progesterone, the substrate prior to DOC, may be accumulated and contribute to thymic involution via thymic stromal cells (Tibbetts *et al.*, 1999). Collectively, metyrapone can contribute to the apoptosis of DP thymocytes by not necessarily inhibiting TCR signaling, but altering the steroid (precursor) microenvironment that has intrinsic apoptotic properties.

King *et al.* also adopted another experimental system to examine the effect of GC hyporesponsiveness on thymocyte development (King *et al.*, 1995). They generated GR TKO mice that express antisense transcripts to the 3' untranslated region (UTR) of GR. Analysis of these mice showed that reduced GC responsiveness substantially lowered the number of TKO DP thymocytes. Furthermore, DP thymocytes from these mice were exquisitely sensitive to activation-induced apoptosis. They also addressed the nature of the TCR repertoire of the TKO mice by immunizing the wild-type, or GR TKO mice with PCC81-104 (the 81-104 C-terminal fragment of pigeon cytochrome c). Previously, it was reported that this peptide is presented by the I-E^{k} MHC molecule and that the majority of responding T cells express TCRs bearing Vα11 and Vβ3 (Davis *et al.*, 1995). While isolated T cells from wild-type immunized mice responded well to PCC81-104, those from TKO mice showed reduced sensitivity. Furthermore, the frequency of Vα11 and Vβ3 T cells was dramatically reduced in the TKO mice. These results suggest that altered GC responsiveness leads to a change in the T-cell repertoire, which produces "holes in the repertoire".

Other antisense GR mice (using the neurofilament promoter) were also analyzed (Morale *et al.*, 1995). Despite presumably being a tissue-specific promoter, these mice showed a reduced GR expression in a broad range of tissues, and increased levels of circulating ACTH and corticosterone, due to decreased negative feedback in the hypothalamo-pituitary-adrenal (HPA) axis. Interestingly, unlike the GR TKO mice, these mice showed no reduction in DP thymocytes. Since the level of circulating GCs on HPA axis is susceptible to feedback regulation and GCs are locally produced in the thymus, it is difficult to clearly see whether GCs affects the thymus by using antisense GR transgenic mice or GC antagonist. However, the obvious inconsistencies that are observed between the two antisense GR mice can be resolved as follows. First, due to the use of different promoters, the expression pattern of GR antisense transgene is quite different from each other - the thymus-restricted expression in the lck-promoter mice, but a more systemic expression in the neurofilament-promoter mice. Therefore, the thymocyte development can be affected differently, according to this disparity in the expression pattern. Moreover, while the effect of adrenal GCs and the HPA axis was relatively minimized in the lck-promoter mice, the widespread reduction in GR signaling in the neurofilament-promoter mice might increase the level of circulating ACTH and the resulting corticosterone, which may have a compensatory effect on GC hyporesponsiveness.

Purton *et al.* recently reported quite incompatible results with previous observations (Purton *et al.*, 2000). They argued that the GR deficiency leads to a loss of responsiveness to GC-induced apoptosis, but does not affect the prenatal thymocyte development by performing FTOC of GR$^{-/-}$ mice. However, GR$^{-/-}$ mice have a systemic defect in GR production; therefore, the feedback regulation of the HPA axis may in some way exert its compensatory effect on the thymus. Therefore, in order to avoid this potential problem, Rag$^{-/-}$ mice should be reconstituted with cells from the fetal liver or bone marrow in either normal or peptide-specific TCR transgenic mice in the GR$^{-/-}$ background. As previously mentioned, thymic development occurs through a narrow selection window, depending on the TCR affinity for the self-peptide/self-MHC complex. A slight change in the TCR or GR signaling pathway may not result in an obvious difference in the CD8/CD4 profile, instead there may be a shift in the TCR chain usage and "holes in repertoire". Therefore, the GR$^{-/-}$ and GR TKO mice should be analyzed in the same fine-tuned experimental system, such as peptide-specific TCR transgenic mice, for an exact interpretation of the experimental data.

Role of GCs in thymic selection

GC sensitivity of thymocytes is developmentally regulated during thymic maturation. While the immature CD4^{+}CD8^{+} DP thymocytes, which constitute approximately 80% of the total thymocytes, are exquisitely sensitive to GC-induced apoptosis, mature CD4^{+}CD8^{-} or CD4^{-}CD8^{+} SP thymocytes are relatively resistant to GCs (Wyllie, 1980; Cohen and Duke, 1984; Hugo *et al.*, 1991; Cohen, 1992; Gruber *et al.*, 1994). Only a small population of DP cells appears to survive and differentiate into SP cells, and finally emigrates from the thymus. Therefore, it appears that the population of immature thymocytes to be positively selected should be rescued from GC-induced death. This is also reflected in the observations that the peak concentration of GCs (0.1~1 μM) in the plasma of a normal mouse can induce apoptosis in its DP thymocytes *in vitro* (Wyllie, 1980; Cohen and Duke, 1984). Then, at least two questions must be addressed to clearly understand the role of GCs in thymic selection: (1) What is the main source of GCs that affect thymic development? (2) What distinguishes

thymocytes that are fated to die by GCs from those that survive GC-induced apoptosis and differentiate into functional T cells?

Traditionally GCs are thought to be synthesized mainly in the adrenal glands from the precursor cholesterol through sequential modifications by members of the cytochrome P450 enzyme family and 3β-hydroxysteroid dehydrogenase. Its synthesis and release from the adrenals is under the control of the HPA axis, ACTH being the major mediator acting on the adrenals (Simpson and Waterman, 1988). GCs synthesized in this way may exert their apoptotic effect in the thymus by being transported in an endocrine manner via blood. However, this cannot be a reasonable explanation for the source of GCs that affect the thymic development. It was reported that the level of circulating GCs is not uniform in the late fetal or neonatal stage, which was considered to be the time when tremendous thymic development occurs. Furthermore, acting as a partial barrier, the placenta that inhibits the transfer of GCs from the mother and placental enzymes in human were shown to inactivate biologically active cortisols (Murphy and Diez d'Aux, 1972). The level of GCs at this stage cannot reach the adult level, because the expression of some steroidogenic enzymes is developmentally regulated (Henning, 1978; Savu *et al.*, 1985). These observations prompted some researchers to investigate whether the thymus itself can produce GCs. Previous studies showed that certain steps of GC synthesis are performed by thymic epithelial cells (TEC), and possibly thymocyte itself (Vacchio *et al.*, 1994; Jenkinson *et al.*, 1999; Pazirandeh *et al.*, 1999). Intensive investigation by Lechner *et al.* has shown that the thymus possesses the entire cohort of enzymes, and cofactors that are required for GC production (Lechner *et al.*, 2000). Interestingly, an intact thymic architecture is necessary for GC production, as shown by the fact that certain steroidogenic enzyme activity could not be detected in the irradiated thymus or in a thymic epithelial cell line. Moreover, the epithelial cell line produced GCs only when in contact with the TCR^+ thymocyte cell line, but not with the TCR^-DN thymocyte clone (Gao *et al.*, 1996). These data raise the intriguing possibility that thymocyte-epithelial interactions may be required for the latter to produce GCs.

Furthermore, some discriminating signals should be provided with thymocytes for a small population of thymocytes to be protected from the apoptotic actions of GCs, and to be shaped into an effective primary T-cell repertoire in the periphery, while the remaining majorities are eliminated in the thymus. It has been suggested that the TCR/CD3 signaling can inhibit the GC-induced apoptosis of T cells. Although the GR and TCR have intrinsic apoptotic properties, when these receptors are activated simultaneously, the T cells survive (Cohen and Duke, 1984; Zacharchuk *et al.*, 1990; Iwata *et al.*, 1991; Liu *et al.*, 1994; Ashwell *et al.*, 1996; Weih *et al.*, 1996; Philips *et al.*, 1997; Vacchio and Ashwell, 2000). Iwata *et al.* suggested a possible role of GCs in the positive selection of specific T cell repertoire by observing that the GC-induced apoptosis of thymocytes and T-cell hybridoma was inhibited by the TCR/CD3-mediated signal (Iwata *et al.*, 1991). They reported that thymocytes were rescued only at a narrow concentration range of each of the antibodies against TCR or CD3. These results suggest that only the thymocytes with proper TCR avidity for self-peptide are rescued from the GC-induced apoptosis during thymic development. Other evidence also supported the idea that the activation of the TCR/CD3 signaling could inhibit the GC-induced apoptosis of T cells. Jamieson and Yamamoto reported that the TCR/CD3 activation of the Ras/Raf/MEK/ERK pathway is necessary and sufficient to protect the 2B4.11 (Fas-negative; $CD3^{high}$) T cell hybridoma, S49 thymoma cell line, and even primary T cells from the GC-induced apoptosis (Jamieson and Yamamoto, 2000). By using the oncogenic Ras mutant, RasV12, partial loss-of-function mutants of RasV12 that selectively activate only one of the Ras downstream effectors and a specific pharmacological inhibitor of each signaling component, they demonstrated that activated Ras alone (independently of TCR triggering) can rescue T cells from GC-induced apoptosis. Among the effectors that are activated by Ras, both Raf and Ral. GDS showed a protective effect against GCs. But, phosphatidylinositol 3-kinase (PI3K), which inhibits the other lymphocyte apoptosis pathway, did not interfere with the GC-induced apoptosis. This report is interesting since it proves that TCR-triggered anti-apoptotic signals rescue thymocytes from GC-induced apoptosis by activating the Ras/MEK/ERK pathway, which is the same pathway as in the thymic positive selection.

As documented, thymocytes undergo two rigorous selection processes across the DP to SP transition. Positive selection directs the survival and differentiation of thymocytes that recognize self-peptides/self-MHC molecules; whereas, negative selection removes the overtly self-reactive thymocyte (Sebzda *et al.*, 1999). Paradoxically, these opposing fates of developing thymocytes are critically determined by the same kind of interaction between αβTCRs and self-peptide fragments that are bound in the groove of the self-MHC molecule (Kaye *et al.*, 1992; Grossman and Singer, 1996; Love *et al.*, 2000). Therefore, what distinguishes the positively selecting signals from the negatively selecting signals is one of the key questions that is still unsettled (Mariathasan *et al.*, 1999). The integration of intricate signaling networks seems to be essential to shape the appropriate TCR repertoire during these selection events. Many studies have addressed the role of the Ras/mitogen-activated protein kinase (MAPK) pathway in the thymocyte selection. Positive selection was impeded in mice that overexpressed the dominant negative components of the Ras/MAPK signaling pathway, such as Ras, Raf, MEK, or both Ras and MEK-1; yet the negative selection was left intact (Alberola-Ila *et al.*, 1995; Swan *et al.*, 1995; Alberola-Ila *et al.*, 1996; O'Shea *et al.*, 1996). Accordingly, retroviral transduction of a constitutive active mutant of MEK-1 enhanced the positive selection, while a pharmacological inhibition of MEK-1 impaired the positive selection.

Moreover, the contribution of several MAPK family members to the thymocyte selection was examined. Positive selection was impaired in ERK1-deficient thymocytes (Pages *et al.*, 1999). Two recent studies also showed that the selective inhibition of ERK activation by impeding the assembly of the TCR/CD3 complex correlated well with the impairment of positive selection. Using transgenic mice that expressed a mutant TCR α-chain-connecting peptide domain (α-CPM), Werlen *et al.* showed that α-CPM controls positive, but not negative selection, by modulating the formation of an intact detergent-insoluble glycolipid-enriched microdomain (DIG)-associated signalsome, which is essential for sustained ERK activity (Werlen *et al.*, 2000). Delgado *et al.* showed that positive selection and the activation of ERK were also markedly impaired in mice that were deficient in CD3δ (Delgado *et al.*, 2000). In both mouse systems, the activation of other MAPKs, such as c-Jun N-terminal kinase (JNK) or p38 kinase, was not dramatically affected. On the other hand, the ablation of JNK2 or inhibition of the JNK pathway in dominant negative JNK1 transgenic mice reduced the *in vivo* deletion of DP thymocytes, which suggests the contribution of JNK in the negative selection (Dong *et al.*, 1998; Rincon *et al.*, 1998; Sabapathy *et al.*, 1999; Dong *et al.*, 2000; Weiss *et al.*, 2000; Sabapathy *et al.*, 2001). Similarly, the attenuation of the p38 pathway also led to impaired negative selection. Recently, the distinct implication of each MAPK pathway in the thymocyte selection was also corroborated by analyses using the mice that were deficient in RasGRP or Grb2. The thymocyte development in the RasGRP-deficient mice was blocked at the DP stage, and the number of mature T cells was dramatically reduced (Dower *et al.*, 2000). DP thymocytes from these mice were also unable to activate the Ras/ERK pathway after TCR crosslinking. In contrast, the haploinsufficiency of Grb2 (a prototypic adaptor protein, which associates with Sos to activate Ras) selectively attenuated JNK and p38 kinase, but not ERK, activation (Gong *et al.*, 2001). In turn, this selective alteration correlated with a reduction in the ability of thymocytes to undergo negative, but not positive, selection. In the homizygous Grb2 mice, only the transient activation of Ras was affected and this was correlated with a defect in the negative selection. The involvement of the TCR activation of the Ras/MEK/ERK pathway in both the positive selection itself and the inhibition of GC-induced apoptosis strongly imply the role of GCs in the positive selection. It seems that crosstalk between the TCR and GR signaling pathways may regulate the survival of thymocytes that undergo the selection process. In other words, DP thymocytes that bear TCRs with subthreshold avidity for self-peptide/self-MHC complexes are programmed to die by a default apoptosis pathway that is termed death by neglect, unless they receive survival signals within about 3.5 days. GCs may play significant roles in this process of cell death. However, thymocytes having TCRs with low-to-intermediate avidity for self-peptide/self-MHC complexes are protected against death by neglect by acquiring GC resistance in response to the TCR activation of the Ras/MEK/ERK pathway. They then differentiate into SP T cells. On the other hand, thymocytes with high avidity undergo activation-induced apoptosis by the full-range activation of ERK, JNK, and p38 kinase. Collectively, the TCR activation of the Ras/MEK/ERK pathway may contribute to the positive selection, at least in part, by rescuing cells that are to be eliminated by GCs during death by neglect. Therefore, the target gene (regulated by the TCR/Ras/ERK pathway to render developing thymocytes GC resistance, although it has yet to be identified) is important in distinguishing the thymocytes that are to survive and undergo further selection from those that will be eliminated by the GC-induced apoptosis.

Another cell surface receptor (Notch family of receptor) has also been known to inhibit GC-induced apoptosis. It has been extensively studied due to its integral roles in regulating cell-fate decisions in many developmental systems, including T cells. Following productive interaction with ligands, such as Delta/Serrate and Jagged, the transmembrane domain of Notch undergoes proteolytic cleavage, releasing its intracellular domain into cytoplasm. After nuclear translocation, the activated Notch can regulate the expression of its target genes through Deltex- and/or CBF-1/Su(H)-mediated signaling (Matsuno *et al.*, 1998; Tamura *et al.*, 1995). Other signaling molecules, which directly bind Notch, include the p50 subunit of NF-κB (in human T cells), EMB-5 (in *C. elegans*), and Dab (in neurons) (Oswald *et al.*, 1998). Some evidence showed that the Notch expression is dynamically regulated in the thymus. Specifically, $CD4^{-}CD8^{-}$ DN thymocytes highly express Notch1, but $CD4^{+}CD8^{+}$ DP thymocytes express low to no Notch1. However, the expression is restored when thymocytes maturate into $CD4^{+}$ or $CD8^{+}$ SP thymocytes (Hasserjian *et al.*, 1996). Interestingly, activated Notch signaling plays protective roles against the GC-induced apoptosis that correlates with the Bcl-2 up-regulation. Deftos *et al.* demonstrated that the retroviral transduction of constitutively active Notch1 protects the thymic lymphoma cell line (AKR1010) and T cell hybridomas (2B4.11) from GC-induced apoptosis (Deftos *et al.*, 1998). Although the CBF-1-binding region of Notch1 (RAM and ankyrin domain) was required for the inhibition of GC-induced apoptosis, the possible implication of Deltex-mediated signaling cannot be excluded. Additionally, thymocytes from transgenic mice that express constitutively active Notch1 became resistant to GC-induced apoptosis. These results, collectively, also strongly suggest that Notch1 activation may rescue thymocytes from death by neglect that is largely mediated by GCs. While activated Notch represses the JNK-mediated activation of E47 (one of the E2A-encoded basic helix-loop-helix (bHLH) transcription factors, through a mechanism requiring Ras in B cells), it is unclear whether a similar crosstalk between the Ras and Notch pathways exist in T cells (Pui *et al.*, 1999). Furthermore, the activation of the Notch signaling pathway has been regarded as a crucial event in the differentiation of DP thymocytes into SP thymocytes

since it promotes thymocytes survival, as well as CD8 differentiation (Robey *et al.*, 1996). The intracellular domain of Notch has been shown to associate with Nur77, a nuclear orphan receptor that is induced upon T cell activation. Nur77 has been implicated in FasL up-regulation. It has been demonstrated (using DO11.10 T cell hybridoma) that activated Notch1 represses the Nur77-mediated transcription of reporter constructs that are driven by the Nur77 DNA-binding response element. This suggests the role of Notch1 in preventing negative selection (Jehn *et al.*, 1999). Many studies have addressed the crosstalk pathway for the inhibition of GC-induced apoptosis by TCR- or Notch-mediated signaling. However, the downstream target molecule that exerts the inhibitory effect on the GC-mediated apoptotic signaling in response to signals that are emanated from these surface receptors is unclear. In the remaining section, we will introduce the SRG3 protein as a key molecule at the converging point in this crosstalk pathway.

SRG3 as a novel modulator in GC-induced apoptosis

Although both the TCR and Notch signaling rescue thymocytes from GC-induced apoptosis, it is not obvious what the downstream effector molecules that render GC resistance in response to these signaling are. The Bcl-2 family of proteins was suggested as one determinant of susceptibility to GCs, because Bcl-2 or Bcl-X_L is highly expressed at the DN or SP stage, which is known to be resistant to GCs. Yet the DP thymocytes, which express them at a low level, are highly sensitive to GCs (Hockenbery *et al.*, 1991; Linette *et al.*, 1994; Ma *et al.*, 1995). They also inhibited GC-induced apoptosis when overexpressed in T cells (Siegel *et al.*, 1992). Furthermore, mature T cells from Bcl-2 homozygous mutant chimeric mice were just as susceptible as DP thymocytes (Nakayama *et al.*, 1993). However, it was suggested that the Bcl-2 up-regulation was a consequence but not a cause of the positive selection in the FTOC analysis of NP34 peptide-specific F5 TCR transgenic mice (Williams and Brady, 2001). The activation of Notch signaling is not always followed by Bcl-2 induction in T cells. Deftos *et al.* showed that the NotchIC9 expression inhibited the GC-induced apoptosis in both the AKR1010 DP thymoma cell line and 2B4.11 T cell hybridoma, but Bcl-2 was up-regulated only in the former cells (Deftos *et al.*, 1998). These results imply that the ability of Notch to render GC resistance may be mediated independently of Bcl-2, at least in the 2B4.11 T cell hybridoma. There may be other general downstream targets besides Bcl-2 that are involved in the Notch1 signaling. Interestingly, the NotchIC overexpression up-regulated the TCR and Deltex expression in AKR1010 cells. Because TCR signaling also inhibits the GC-induced apoptosis, it is also possible that Notch renders thymocytes a resistance to GCs by facilitating the inhibitory signal against the GC-induced apoptosis that is triggered by TCR engagement. Furthermore, as stated previously, although it was suggested that the CBF-1 pathway is important for the Notch inhibition of the GC-induced apoptosis by introducing a point mutation into its binding sites in NotchIC, the direct transcriptional regulation by the Notch via Deltex-mediated pathway cannot be understated.

The murine SWI3-related gene (SRG3) was cloned by subtraction between the thymic and lymph node RNAs in order to identify molecules that were highly expressed in the thymus (Jeon *et al.*, 1997). It is a murine homolog of human BAF155 and yeast SWI3. The expression pattern of SRG3 is correlated well with the GC sensitivity of developing thymocytes - the increase in GC resistance, but the decrease in the SRG3 expression across the DP to SP transition. These results strongly imply the role of SRG3 in the GC-induced apoptosis of DP thymocytes.

In order to address this possibility, Jeon *et al.* introduced the antisense SRG3 transcript into the S49.1 thymoma cell line. Down-regulation of the SRG3 expression resulted in the reduction in the GC-induced apoptosis. On the other hand, when SRG3 was overexpressed in the EL4 $CD4^+$ T cell line, which is relatively resistant to GCs and normally expresses a low level of SRG3, these cells became sensitive to GCs (Jeon *et al.*, 1997). The clear correlation between the expression level of SRG3 and GC sensitivity was also confirmed *in vivo*. When antisense SRG3 transcripts were overexpressed under the control of the lck proximal promoter, thymocytes from these transgenic mice (*lck*-αSRG3 mice) expressed SRG3 by a half, while the GR expression was left intact and became resistant to the GC-induced apoptosis (Choi *et al.*, 2001). Moreover, 3-4 week-old *lck*-αSRG3 mice showed a slight increase in thymocyte numbers, possibly due to the reduction in the GC-induced apoptosis of DP thymocytes. On the other hand, when SRG3 was overexpressed under the control of the human CD2 promoter, the SRG3 expression in the lymph node increased (approximately 2 fold). This rendered more sensitivity to GCs on the lymph node T cells, which were relatively resistant to GCs in control mice.

Intriguingly, SRG3 associates with GR *in vivo*, depending on the expression level of SRG3. When thymic extracts were analyzed by glycerol gradient or gel filtration, SRG3 was detected in the 600 KDa fraction together with GR, but independently of the SWI/SNF complex, which was detected in the 2 MDa fraction (Han and Seong, unpublished data). The subcellular localization of SRG3 and BRG1 was also assayed by confocal microscopy in various cell lines. SRG3 and BRG1 were both detected in the nucleus of the NIH3T3 fibroblast cells. However, SRG3 and GR were observed as a complex in the cytoplasm of the 16610D9 DP thymocyte cell line and the EL4 $CD4^+$ T cell line in the absence of GCs. When GCs was treated, the nuclear localization of GR was readily detected, but the majority of SRG3 still remained in the cytoplasm. BRG1 was mainly localized in the nucleus, as expected, independently of the presence of GCs (Han and Seong, manuscript in preparation). These confocal

microscopy data implied the cell type-specific localization of SRG3, as well as the possibility that SRG3 may play a unique role in the GC-induced apoptosis by associating with GR in T cells. Furthermore, the complex formation between SRG3 and GR was functionally correlated with GC sensitivity. The lymph node T cells in the hCD2-SRG3 overexpression mice showed an increase in the SRG3/GR complex and more sensitivity to the GC-induced apoptosis compared to the wild-type cells. However, SRG3 seems to specifically act as a potentiator of the GC-induced apoptosis, but not other types of lymphocyte apoptosis since there was no effect of the modified SRG3 expression on the FAS- or staurosporine-induced apoptosis (Han *et al.*, 2001). SRG3 was shown to regulate the transcriptional activity of GR. The reduction of the SRG3 expression by the expression of the antisense SRG3 transcript in the S49.1 thymoma cell line lowered the GRE-mediated reporter activity by inhibiting the GR/SRG3 complex formation. Conversely, the increase in the SRG3 expression in the EL4 mature T cell line led to an increase in the GR/SRG3 protein complex, leading to an increase in the reporter activity (Jeon and Seong, unpublished data). Additionally, 89 amino acid fragments of SRG3 inhibited the formation of the SRG3/GR complex in a dose-dependent manner by acting dominant negatively. The ectopic overexpression of this dominant negative fragment inhibited the GR-mediated transcription, as well as the GC-induced apoptosis in the S49.1 thymoma cell line (Han *et al.*, 2001). Collectively, these results clearly show that the expression level of SRG3 is a key determinant of the SRG3/GR complex formation and thus the sensitivity to the GC-induced apoptosis of T cells by regulating the GR-mediated transcription.

A previous report showed that the SWI/SNF complex, including BRG1, is essential for the GR-mediated transcription in the T47D human breast cancer cell line (Fryer and Archer, 1998). However, the data from size fractionation and confocal microscopy, as described previously, suggested a novel possibility that SRG3 may play a role in the GC-induced apoptosis, independently of the BRG1-containing SWI/SNF complex. This discrepancy can be resolved in terms of serial binding of different protein components into the complex. Specifically, GR exists as a GR/SRG3 protein complex in the cytosol of thymocyte and GC induces the translocation of the GR/SRG3 complex into the nucleus, binding to the target DNAs, followed by subsequent recruitment of other SWI/SNF components.

Recently, the possibility that the expression of SRG3 might be regulated during thymocyte selection was also directly addressed. A recent report demonstrated that the expression of SRG3 decreased during thymocyte development, especially during positive selection (Choi *et al.*, 2001). In this report, two thymocyte populations, $CD3^{lo}CD69^{-}$ (presumably before the positive selection) and $CD3^{hi}CD69^{+}$ (after the positive selection) cells, were sorted from thymi of C57BL/6J mice using flow cytometry. The CD69 and TCR/CD3 expressions were known to be induced after positive selection. In order to estimate the expression levels of SRG3 in the two populations, semi-quantitative competitive RT-PCR was performed with total RNAs that were isolated from each sorted population. The expression level of the SRG3 gene was reduced to one third in the $CD3^{hi}CD69^{+}$ thymocyte population, compared to $CD3^{lo}CD69^{-}$ thymocytes population. Furthermore, anti-CD3ε antibody-mediated or PMA/Ionomycin-mediated activation of S49.1 or 16610D9 thymoma cells resulted in a similar down-regulation of the SRG3 expression (Ko and Seong, manuscript in preparation). Since similar results were obtained by using KCIT1-8.5 T hybridoma cells defective in Fas induction upon TCR stimulation, the TCR down-regulation of the SRG3 protein is independent of activation-induced apoptosis. Activated Ras alone (instead of TCR activation) lowered the SRG3 protein expression, as well as its promoter activity (Ko and Seong, manuscript in preparation). These data implied that the SRG3 expression is down-regulated by the TCR/CD3 signaling during the positive selection of DP thymocytes.

The same semi-quantitative RT PCR with RNA from thymocytes of the NotchIC-9 transgenic mice (expressing the activated form of Notch1 in the thymus specifically) showed that the level of the SRG3 expression in the $CD3^{lo}CD69^{-}$ population was even lower than that in the $CD3^{hi}CD69^{+}$ thymocyte population of normal mice. Similarly, DP thymocytes of NotchIC-9 mice showed a reduction in the SRG3 protein, but no difference in the GR expression when analyzed by Western blotting. More importantly, the restoration of the SRG3 expression level by mating the SRG3 overexpression mice with NotchIC transgenic mice yielded an increased GC-sensitivity of DP thymocytes (Choi *et al.*, 2001).

The fact that the SRG3 expression is down-regulated by TCR/CD3 and NotchIC signaling strongly implies that the developing thymocytes can acquire GC resistance by down-regulating SRG3 in response to the TCR/CD3 and/or Notch signaling. Particularly during the positive selection, DP thymocytes that express a low level of SRG3 in response to these signaling may become resistant to GCs and differentiate into functional T cells. However, in the absence of these signaling, DP thymocytes still express a high level of SRG3, and thus are eliminated by the GC-induced apoptosis.

However, the specific role of GCs in thymocyte development is still debatable. Most of studies on the role of GCs in thymocyte development were based on strategies that directly modify the level of GCs or GR (King *et al.*, 1995; Sacedon *et al.*, 1999; Lu *et al.*, 2000; Purton *et al.*, 2000). However, transgenic mice that overexpress SRG3 or the antisense SRG3 transcript can provide a useful alternative system in order to understand the role of GCs in thymocyte development, because SRG3 is specifically involved in the regulation of the GC-induced apoptosis of thymocytes without altering the GR expression or GC production. This may exclude the possible compensatory effect of the HPA axis on the thymus, which may contribute to seemingly contradictory results on the role of GCs observed thus far. Currently, an

analysis of the thymocytes that bear peptide-specific TCR in these transgenic mice is underway.

Concluding Remarks

GCs play a critical role in thymocyte development. They cause T cells to die by GC-induced apoptosis, or regulate the window of thymocyte selection. The GC-mediated signaling intricately communicates with other signaling events, such as TCR- or Notch-mediated signaling, to achieve thymocyte differentiation. A few molecules were discussed as candidate molecules that mediate the crosstalk between these pathways. Among them, SRG3 was discussed in great detail. Unlike the pan-anti-apoptotic Bcl-2 protein in numerous systems, SRG3 is a highly expressed protein in the thymus that plays a specific role in thymic apoptosis. Manipulation of the SRG3 expression levels gives a more specific effect on the GC-induced thymocyte apoptosis without modifying the level of GR or GCs, because the effect is independent of the potential compensatory effect of the HPA axis. This opens the possibility for SRG3 as a useful therapeutic target. Many advances have been made in understanding the thymic development, but an ultimate understanding will be achieved by dissecting the intricate regulation of the signaling pathways in the thymus.

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