Autocrine Action of Adiponectin on Human Fat Cells Prevents the Release of Insulin Resistance–Inducing Factors

Daniela Dietze-Schroeder, Henrike Sell, Mathias Uhlig, Marlis Koenen, and Jürgen Eckel

The adipocyte hormone adiponectin is negatively correlated with obesity and insulin resistance and may exert an important antidiabetes function. In this study, primary human skeletal muscle cells were cocultured with human fat cells or incubated with adipocyte-conditioned medium in the presence or absence of the globular domain of adiponectin (gAcrp30) to analyze its capacity to restore normal insulin signaling in the muscle cells. Human skeletal muscle cells cocultured with adipocytes or treated with adipocyte-conditioned medium showed an impaired Akt and glycogen synthase kinase 3 serine phosphorylation in response to insulin. Furthermore, insulin-stimulated GLUT4 translocation was reduced by adipocyte-conditioned medium. Impaired insulin signaling was normalized upon addition of gAcrp30 to the coculture. Further, adipocyte-conditioned medium generated in the presence of gAcrp30 was unable to perturb insulin-stimulated Akt phosphorylation. Concomitant addition of gAcrp30 and adipocyte-conditioned medium to the myocytes failed to restore normal insulin action. Protein array analysis of adipocyte-conditioned medium indicated that the secretion of at least eight different cytokines was diminished in response to gAcrp30. We therefore suggest that adiponectin operates as a key regulator of adipocyte secretory function. This autocrine action may prevent the induction of skeletal muscle insulin resistance and may partly explain the antidiabetes action of this hormone. Diabetes 54: 2003–2011, 2005

It is now well accepted that adipose tissue represents a major secretory and endocrine active organ producing a variety of factors that may regulate energy metabolism and insulin sensitivity (1). Increased adipose tissue mass, especially in the visceral compartment, is associated with insulin resistance, hyperglycemia, dyslipidemia, hypertension, and other components of the metabolic syndrome (2,3) and represents one of the major risk factors for the development of type 2 diabetes (4–6). Adipocytes from obese subjects exhibit an altered endocrine function and secretory profile leading to an increased release of adipocytokines and proinflammatory molecules including tumor necrosis factor (TNF)α, interleukin (IL)-6, angiotensinogen, and resistin (7,8). Some of these factors play a key role in the induction of skeletal muscle insulin resistance in rodents (9); however, their precise role in humans remains controversial (10). Adiponectin or adipocyte complement–related protein of 30 kDa (Acrp30) is the only known adipocytokine in which plasma levels are decreased in obesity and type 2 diabetes (11–13). Low adiponectin plasma levels are good indicators of insulin resistance and the development of diabetes (14,15). In studies with obese and diabetic rodents, it was further shown that intravenous application of adiponectin leads to normalized insulin sensitivity (16,17). Many studies focused on the physiological importance of adiponectin and support the notion of an antidiabetes action of this hormone. However, the cellular and molecular basis of this effect remains poorly understood, and little is known about the role of adiponectin in the cross talk between adipose tissue and skeletal muscle. Known effects of adiponectin in vitro include an antiapoptotic action on pancreatic β-cells (18) and an anti-inflammatory and vasoprotective function in vascular endothelial cells (12,19). Furthermore, adiponectin secretion by 3T3 adipocytes is decreased by proinflammatory adipocytokines like IL-6 (20).

In the present study, we took advantage of our recently described coculture model of human adipocytes and skeletal muscle cells (10) to elucidate the role of adiponectin in the cross talk between adipose tissue and skeletal muscle. We report here that adiponectin prevents the induction of muscle insulin resistance by reducing the release of fat cell secretory products. These factors include IL-6, IL-8, growth-regulated oncogene (GRO)-α, monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1α and -1β, and tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2. We suggest that adiponectin acts as a key regulator of adipocyte secretory function and that this autocrine action may contribute to the antidiabetes effect of this hormone.

RESEARCH DESIGN AND METHODS

BSA (fraction V, fatty acid free) was obtained from Boehringer (Mannheim, Germany). Reagents for SDS-PAGE were supplied by Amersham Pharmacia Biotech.
Biotech (Braunschweig, Germany) and Sigma (München, Germany). The recombinant COOH-terminal globular domain of adiponectin and full-length adiponectin were products from Tebu (Offenbach, Germany). Polyclonal antibodies anti-β-polysaccharide synthase kinase (GSK) 3α/β (Ser21/9), anti-phospho-Akt (Ser473), and anti-Akt were supplied by Cell Signaling Technology (Frankfurt, Germany). Anti-GSK3α/β was from Stressgene (Victoria, Canada). Cytokine protein arrays (RayBio Antibody Array C Series 1000) were purchased from RayBiotech (Norcross, GA). Collagenase CLS type 1 was obtained from Worthington (Freehold, NJ), and culture media were obtained from Gibco (Berlin, Germany). The cytokines IL-6, IL-8, MCP-1, MIP-1α, and MIP-1β were purchased from Hölzel Diagnostics (Cologne, Germany). Primary human skeletal muscle cells and supplement pack for growth were obtained from PromoCell (Heidelberg, Germany). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody was from Promega (Mannheim, Germany). Cytokine protein arrays were generated by culturing adipocytes in absence or presence of 5 mmol/l global domain of adiponectin (gAdip30) from different fat donors (n = 5) were hybridized with the array membranes according to the protocol supplied by the manufacturer. Briefly, membranes were blocked by a Pierce (Rockford, IL). Signals were visualized and evaluated on a LumiImager (Roche Diagnostics). The signal intensity was normalized to internal positive signals on the membrane. For each fat donor, conditioned medium generated in presence of 5 mmol/l gAdip30 and control medium without addition of adiponectin was analyzed at the same time and cytokine signals compared. Cytokine concentrations in conditioned medium were determined with the same cytokine array. A mix of the five measured cytokines was analyzed twice using different concentrations to assure linearity of the assay. Absolute cytokine concentrations were calculated based on the calibration curve. The level of adiponectin in adipocyte-conditioned medium was determined using an enzyme-linked immunosorbent assay kit from B-Bridge International (Sunnyvale, CA). The assay was performed as recommended by the manufacturer using duplicate samples for all determinations.

**Results**

Adiponectin ameliorates impairment of insulin signaling in skeletal muscle cells cocultured with human adipocytes. As we were previously able to demonstrate, coculture of human adipocytes and human skeletal muscle cells leads to a rapid disturbance of insulin signaling in the muscle cell, as seen by a prominent reduction in the activity of insulin receptor substrate–1/Akt phosphorylation (10). As presented in Fig. 1A, this impairment of insulin signaling could also be detected further downstream at the level of GSK3. Cytokine conditions increased basal phosphorylation of GSK3 and concomitantly reduced the effect of insulin on this process. Thus, the fold stimulation was reduced from 2.5 ± 0.2 to 1.6 ± 0.3 and from 3.7 ± 0.9 to 1.9 ± 0.4 (n = 12) for GSK3α and GSK3β, respectively. Most importantly, adipocyte-conditioned medium, which mimicks the coculture condition (22), profoundly reduced insulin-stimulated GLUT4 translocation in human skeletal muscle cells (Fig. 1B). This assay detects the movement of GLUT4 to the cell surface, a major effect of insulin in this tissue. We therefore conclude that adiponectin ameliorates impairment of insulin signaling in skeletal muscle cells cocultured with human adipocytes.

**Primer and RT-PCR.** Total RNA was extracted from differentiated human skeletal muscle cells and adipocytes by using Trizol (Roche Diagnostics) following the manufacturer’s protocol. The reverse transcription reaction and the following PCR were performed with the One Step RT-PCR kit (Qiagen, Hilden, Germany), as previously described (24).

**Assay of GLUT4 translocation.** Recombinant, replication-defective adenoviral vectors were generated with the AdenoVator system from QBiogene (Heidelberg, Germany). Three days after start of differentiation, skeletal muscle cells were infected with recombinant adenoviruses encoding GLUT4myc and used for analysis after an additional 48 h incubation. After stimulation with 10−7 mol/l insulin for 30 min, GLUT4 translocation was measured based on the protocol described by Kanai et al. (25).
cyte-derived factors impair both insulin signaling and downstream insulin action in the myocytes.

To investigate whether adiponectin has the capacity to prevent the induction of impaired myocyte insulin signaling under coculture conditions, adipocytes and skeletal muscle cells were cocultured for 48 h in the absence or presence of 10 nmol/l gAcrp30. After coculture, skeletal muscle cells were acutely stimulated with insulin, and downstream signaling was assessed at the level of the serine/threonine kinase Akt, a key mediator of insulin action on glucose transport and glycogen synthesis (26). As reported earlier, coculture leads to a significant reduction of insulin-induced serine phosphorylation of Akt without affecting basal phosphorylation or expression level of the kinase (Fig. 2A). As can be seen from the data, the presence of adiponectin during the coculture period clearly prevents the impairment of insulin signaling in the muscle cells. Under these conditions, Akt serine phosphorylation after acute insulin stimulation was not significantly different from the control situation (Fig. 2A).

To check if the two cell types express the receptors for adiponectin, we analyzed the cells using RT-PCR. Our results show that in vitro–differentiated human skeletal muscle cells and adipocytes express both adiponectin receptor 1 and 2 (Fig. 2B). As shown in Fig. 2C, adiponectin has no direct effect on insulin-stimulated Akt phosphorylation in skeletal muscle cells. Thus, adiponectin-mediated prevention of impaired insulin signaling in the myocytes (Fig. 2A) cannot be explained by augmented insulin responsiveness of Akt phosphorylation in these cells.

**Autocrine action of adiponectin on human adipocytes prevents the release of insulin resistance–inducing...**
factors. We next addressed the question of whether skeletal muscle cells incubated with adipocyte-conditioned medium and adiponectin are protected from impaired insulin signaling. Adipocyte-conditioned medium impaired insulin signaling in skeletal muscle cells at the level of Akt serine phosphorylation by 50–60% without affecting the expression level of this enzyme (Fig. 3). Concomitant incubation of muscle cells with adiponectin and adipocyte-conditioned medium did not prevent impairment of insulin signaling, making it likely that the results observed in the coculture reflect adiponectin action on the fat cells.

To provide evidence for an autocrine action of adiponectin, fat cells from the same fat cell donors were cultured for 48 h in the absence or presence of 5 nmol/l gAcrp30 and the conditioned medium was processed for experiments with myotubes. As presented in Fig. 4, impairment of insulin-stimulated Akt phosphorylation by adipocyte-conditioned medium was nearly abolished when generated in the presence of adiponectin. This experiment was repeated with the same result using full-length adiponectin. Furthermore, adipocyte-conditioned medium induced a significant impairment of insulin signaling downstream of Akt (69.9 ± 3.5 and 86.4 ± 4.1% [n = 4] of insulin-stimulated control for GSK3α and -β phosphorylation, respectively). This inhibitory action was completely abolished when testing adipocyte-conditioned medium generated in the presence of gAcrp30 (99.2 ± 8.9 and 100.9 ± 2.9% of insulin-stimulated control for phospho-GSK3α and -β, respectively). These observations suggest that adiponectin can counteract induction of skeletal muscle insulin resistance by autocrine action on adipocytes. This notion is supported by the determination of endogenous adiponectin in adipocyte-conditioned media. As presented in Table 1, a very low concentration of adiponectin was associated with inhibitory activity on insulin signaling in skeletal muscle cells. This inhibitory activity was not found in conditioned media with 10- to 20-fold higher endogenous adiponectin levels (Table 1). It is worth noting that the addition of gAcrp30 for 48 h did not alter the adiponectin release by the adipocytes. Furthermore, we observed lower MCP-1, MIP-1α, and MIP-1β concentrations (reduced to 40, 30, and 10%, respectively) in conditioned media with higher endogenous adiponectin that were not used for further experiments. It should be noted that low adiponectin is unrelated to any clinical background of the donors, since adipose tissue was always obtained from healthy subjects.
Adiponectin downregulates cytokine secretion from human adipocytes. To identify adipocytokines that are secreted by differentiated human adipocytes and regulated by adiponectin, we analyzed adipocyte-conditioned medium by a cytokine protein array detecting 120 different cytokines. In conditioned medium from five different fat donors, the following cytokines were identified: adiponectin, GRO-α, hepatocyte growth factor (HGF), IGF-binding protein (IGFBP)-3, IL-6, IL-8, MCP-1, macrophage-derived chemokine, MIP-1α, MIP-1β, osteoprotegerin, soluble TNF receptor 2 (sTNFR 2), TIMP-1, and TIMP-2. In conditioned media from some individuals we also detected angiogenin, fibroblast growth factor-9, intercellular adhesion molecule-1, neutrophil activating peptide-2, TNFα, TNFβ, and plasminogen activator receptor. To evaluate the effect of adiponectin treatment on secretion of adipocytokines, we analyzed adipocyte-conditioned medium generated in the presence of 5 nmol/l gAcrp30 in comparison to the respective control adipocyte-conditioned medium (Fig. 5A). Macrophage-derived chemokine, IGFBP-3, and HGF secretion was not affected by adiponectin treatment. However, adiponectin reduced IL-6, IL-8, and GRO-α secretion by 50–60% (Fig. 5B). Several members of the small inducible cytokine family such as MCP-1, MIP-1α, and MIP-1β were also significantly reduced. From the two TNF-related proteins, only osteoprotegerin was significantly reduced. TIMP-1 and TIMP-2, two cytokines that are thought to play a role in adipocyte differentiation, were also significantly reduced in medium generated in the presence of adiponectin (Fig. 5B). A comparable reduction in adipokine secretion was also observed in response to 5 nmol/l full-length adiponectin (percent of untreated control: IL-6 52 ± 3, IL-8 52 ± 14, MCP-1 55 ± 8, MIP-1α 61 ± 15, MIP-1β 66 ± 12, osteoprotegerin 53 ± 14, TIMP-1 39 ± 7, TIMP-2 44 ± 13; n = 3). The concentrations of several adiponectin-regulated cytokines in the supernatant of cultured human adipocytes are presented in Table 2. As reported earlier, a very high amount of IL-6 is released by these cells (27), whereas IL-8 and MCP-1 are present at 50- and 200-fold concentrations above the physiological level, in excellent agreement with recent observations (28,29).

DISCUSSION

Adipocyte-derived factors such as TNFα and IL-6 are significantly increased in obesity and are good predictors of the development of type 2 diabetes (30,31). Obesity thereby contributes to a proinflammatory milieu, and it is now recognized that adipose tissue functions as an endocrine organ secreting a variety of proinflammatory factors. Adiponectin is the only adipocytokine known to be downregulated in obesity; however, little is known about the role of adiponectin in the cross talk between adipose tissue and skeletal muscle itself. We report here for the first time on adiponectin acting as an autocrine regulator of adipokine secretion of the human fat cell. By decreasing cytokine release by the adipocyte, adiponectin prevents the impairment of insulin signaling in a coculture model of...
human adipocytes and skeletal muscle cells. This is in accordance with the view that the cross talk between inflammatory and metabolic signaling pathways may elicit insulin resistance and extends this concept to human skeletal muscle. Adiponectin secretion is very low by in vitro–differentiated human adipocytes in accordance with studies using freshly isolated mature adipocytes in long-term culture (32). Furthermore, we found here that the low level of adiponectin correlated with the release of insulin resistance–inducing factors by adipocytes. Therefore, the coculture represents an ideal model of adiponectin deficiency to study the role of adiponectin in the cross talk between fat and muscle. It is worth noting that most of our experiments were performed using the globular head domain of adiponectin. Adiponectin prevails in serum as a trimer, hexamer, or high–molecular weight form, with controversial results being reported on the biological activity of these isoforms (33). The globular head domain of adiponectin is generated by leukocyte elastase (34) and became detectable in human plasma at low abundance when using immunoprecipitation with a globular head–specific antiserum (35). Furthermore, the head domain binds to adiponectin receptors 1 and 2, and transgenic expression protects ob/ob mice from diabetes (36). However, the physiological relevance of gAcrp30 remains controversial, since other studies failed to detect it in human serum samples using nonreducing and non–heat-denaturing SDS-PAGE (37) or velocity sedimentation (38). Nevertheless, the high efficiency of gAcrp30 in animal studies highlights the potential role of this molecule for future drug development.

Earlier studies focused on adiponectin action in skeletal muscle and adipose tissue. Adiponectin was found to activate AMP kinase in skeletal muscle (39) depending on the muscle type (40). In myocytes, adiponectin may also activate the nuclear factor-κB pathway (41). Furthermore, in this tissue, adiponectin treatment leads to enhanced fat oxidation and glucose transport. Adiponectin expression in adipocytes is regulated by various compounds such as TNFα and growth hormone, which also influence insulin sensitivity (42,43), and adiponectin treatment leads to increased glucose uptake (44). In the current investigation, we focused on the cross talk between these two tissues, which are both critical players regarding insulin resistance and diabetes. We propose a model of autocrine/paracrine adiponectin action on adipose tissue as a pivotal determinant of muscle insulin sensitivity. In this respect, it was shown here that adiponectin had no effect on insulin signaling at the level of Akt in the muscle cells, neither alone nor in combination with adipocyte-conditioned medium, although these cells express both types of adiponectin receptors. Thus, in our model the beneficial effect of adiponectin on muscle insulin signaling is clearly adipocyte dependent since 1) it was observed upon addition to the coculture and 2) the insulin resistance–inducing effect of conditioned medium was ameliorated when generated in the presence of adiponectin. A paracrine effect of adiponectin on fat cell formation in bone marrow (45) and fat cell differentiation (46) was already proposed. Furthermore, adiponectin is considered an autocrine regulator of energy metabolism (47). An autocrine regulation of cytokine secretion in adipocytes adds a new mechanism to the pleiotropic action potential of adiponectin.

In this study, we identified several cytokines exhibiting adiponectin-regulated secretion from differentiated human adipocytes. Some of these adiponectin-regulated cytokines such as IL-6, IL-8, and MCP-1 are known to be related to obesity and diabetes. Others such as GRO-α, TIMP-1

### TABLE 1

<table>
<thead>
<tr>
<th>Conditioned medium</th>
<th>Adiponectin concentration (ng/ml)</th>
<th>Inhibition of Akt phosphorylation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM 1</td>
<td>1.13</td>
<td>55 ± 5 (6)</td>
</tr>
<tr>
<td>CM 2</td>
<td>0.43</td>
<td>57 ± 12 (6)</td>
</tr>
<tr>
<td>CM 3</td>
<td>0.83</td>
<td>29 ± 6 (3)</td>
</tr>
<tr>
<td>CM 4</td>
<td>0.74</td>
<td>43 ± 7 (5)</td>
</tr>
<tr>
<td>CM 5</td>
<td>0.74</td>
<td>30 ± 10 (5)</td>
</tr>
<tr>
<td>CM 6</td>
<td>0.80</td>
<td>38 ± 4 (2)</td>
</tr>
<tr>
<td>CM 7</td>
<td>9.06</td>
<td>ND</td>
</tr>
<tr>
<td>CM 8</td>
<td>17.40</td>
<td>ND</td>
</tr>
</tbody>
</table>

Data are means ± SE (no. of experiments). Conditioned medium (CM) of eight different donors was generated as outlined in RESEARCH DESIGN AND METHODS. Duplicate samples were analyzed using an adiponectin enzyme-linked immunosorbent assay kit. Inhibition of insulin-stimulated Akt phosphorylation was determined as outlined in RESEARCH DESIGN AND METHODS. ND, inhibition not detectable.
and -2, and MIP-1 are related to inflammation and tissue remodeling, but their relation to adipose tissue, obesity, and diabetes is less clear. IL-6, IL-8, and MCP-1 are well known to be induced in the obese state in humans and rodents (48–51). IL-6 is expressed both by adipose tissue and skeletal muscle (52). Elevated plasma concentrations of these adipokines in obese and insulin-resistant patients may contribute to the insulin-resistant state observed in obesity. Interestingly, adiponectin concentrations are inversely correlated to IL-6 plasma concentrations, insulin sensitivity, and obesity in full agreement with our findings (48). However, the role of IL-6 in skeletal muscle remains controversial with publications both supporting and not supporting involvement of IL-6 in impaired insulin action (53). Our recent study showed that only extremely high concentrations of IL-6 produced a slight impairment of insulin signaling in human skeletal muscle cells, making it unlikely that IL-6 alone is sufficient to induce muscle insulin resistance (27). IL-8 expression is increased by proinflammatory cytokines such as TNFα and IL-1, it may be involved in obesity-related complications. The potent reduction of IL-8 secretion by adiponectin shown here supports the notion (49) that this cytokine may play a role in the induction of insulin resistance, most likely in concert with other adipokines.

We show here for the first time that GRO-α is secreted...
from human adipocytes. This cytokine is structurally related to IL-8 and considered to attract neutrophils to the site of inflammation and may play a role in inflammation, angiogenesis and tumorigenesis (55). Its role in adipose tissue and the regulation of insulin sensitivity needs to be defined. MCP-1 is clearly associated with the obese state (50,51). Its overexpression, especially in epicardial adipose tissue, is thought to increase the inflammatory burden of arteries (56). In adipocytes, MCP-1 expression is increased by TNFα, insulin, growth hormone, and IL-6 (57). Treatment of 3T3-L1 adipocytes with MCP-1 was found to impair glucose uptake, indicating that this cytokine may contribute to the pathogenesis of insulin resistance (50), although the effect of MCP-1 on skeletal muscle insulin action needs to be established.

TIMP-1 and -2, in combination with the matrix metalloproteinases, exert key functions in extracellular matrix remodeling. As for TIMP-1, it was shown to be strongly induced in obesity (58). Matrix metalloproteinases and TIMP activity may be essential for adipogenesis since changes in cell-matrix interaction must accompany adipocyte hypertrophy as well as recruitment and differentiation of adipocyte precursors. This is supported by the observation that TIMP-1 knockout mice are less sensitive to the induction of obesity (59). By decreasing secretion of TIMPs, adiponectin may decrease adipocyte hypertrophy and fat accumulation. Thus, adiponectin could directly contribute to tissue remodeling by increasing the number of smaller adipocytes which are known to better retain free fatty acids and contribute to increased insulin sensitivity. MIP-1α is expressed by human adipocytes, but its secretion decreases upon differentiation (28), a feature shared with IL-8. Regulation of MIP-1α by adiponectin may also contribute to decreased adipocyte hypertrophy. Thus, in addition to regulating the release of inflammatory cytokines that may interfere with insulin signaling in the muscle cell, adiponectin may also exert its anti-inflammatory action by regulating fat cell differentiation and growth.

In summary, our data show that adiponectin acts as a key regulator of cytokine secretion in adipose tissue. We therefore suggest that cytokines regulated by adiponectin may represent a molecular link between obesity and skeletal muscle insulin resistance. These adipocytokines are involved in inflammation, tissue remodeling, and angiogenesis, but their role in obesity and the development of skeletal muscle insulin resistance needs to be further analyzed.

ACKNOWLEDGMENTS

This work was supported by the Ministerium für Wissenschaft und Forschung des Landes Nordrhein-Westfalen, the Bundesministerium für Gesundheit, European Union COST Action B17, the Deutsche Forschungsgemeinschaft (FOR 441 and EC 64/11-1), and the Foundation “Das zuckerkrankes Kind.”

We thank Prof. R. Olbrisch and his team, the Department of Plastic Surgery, Florence Nightingale Hospital, Düsseldorf, for support in obtaining adipose tissue samples. The secretarial assistance of Birgit Hurow is gratefully acknowledged.

REFERENCES

28. Gerhardt CC, Romero IA, Cancello R, Camoin L, Strosberg AD: Chemo...
29. Wang B, Jenkins JR, Trayhurn P: Expression and secretion of inflamma...
30. Pradhan AD, Manson JE, Rifai N, Buring JE, Ridker PM: C-reactive protein, ...
31. Waki H, Yamauchi T, Kamon J, Ito Y, Uchida S, Kita S, Hara K, Hada Y, ...
32. Gerhardt CC, Romero IA, Cancelli R, Camoin L, Strosberg AD: Chemokines...
33. Waki H, Yamauchi T, Kamon J, Ito Y, Uchida S, Kita S, Hara K, Hada Y, ...
34. Waki H, Yamauchi T, Kamon J, Ito Y, Uchida S, Kita S, Hara K, Hada Y, ...
35. Waki H, Yamauchi T, Kamon J, Ito Y, Uchida S, Kita S, Hara K, Hada Y, ...
36. Yamauchi T, Kamon J, Waki H, Imai Y, Shimozawa N, Hioki K, Uchida S, ...
37. Waki H, Yamauchi T, Kamon J, Ito Y, Uchida S, Kita S, Hara K, Hada Y, ...