Transcriptional regulation of IL-6 in bile duct epithelia by extracellular ATP

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The inflammatory cytokine IL-6 is potently upregulated in a variety of forms of liver injury. Bile duct epithelia (BDE) are critical sources of IL-6 in the injured liver (27, 39). Recent studies have shown that this upregulation is necessary for survival. In the absence of IL-6 or its receptor, viability after injury is markedly diminished (10–12). Decreased survival in the absence of IL-6 is due to inadequate proliferation of BDE, which form a pool of precursors for hepatic parenchymal cells. Although the importance of IL-6 is established, the downstream signals through which IL-6 acts are less well understood.

In recent years, we have reported that loss of expression of the ecto-ATPase NTPDase2 is a critical link between liver fibrogenesis and proliferation of BDE (15, 17, 24, 41). More recently, we observed that release of IL-6 by BDE potently downregulates NTPDase2 expression by neighboring portal fibroblasts (PF). Loss of NTPDase2 leads to induction of BDE proliferation. Thus IL-6 upregulation is a critical step in the paracrine loop between BDE and PF. However, the mechanisms leading to bile ductular IL-6 upregulation are unknown.

BDE express P2Y receptors linked to a variety of downstream processes (18, 36, 43). P2Y receptors are plasma membrane G protein-coupled receptors for extracellular nucleotides (35). Although initial descriptions of P2Y receptors suggested that receptor activation was linked to cytosolic Ca2+ (Ca2+2) signals, novel receptors that link to cAMP have recently been described (7, 13, 28). We investigated whether activation of P2Y receptors regulated IL-6 transcription by BDE. Here we demonstrate that extracellular ATP induces IL-6 transcription by BDE in a mechanism involving Ca2+2 and cAMP signals. Furthermore, these two messengers act synergistically via a cAMP-response element (CRE) in the IL-6 promoter.

MATERIALS AND METHODS

Reagents. Adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), uridine triphosphate (UTP), uridine diphosphate (UDP), thapsigargin, and suramin were purchased from Sigma (St. Louis, MO). Adenosine 5’-(gamma-thio)triphosphate (ATPγS), 1,2-bis-(o-aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid, tetracetoxymethyl ester (BAPTA/AM), dibutyryl-cyclic AMP (db-cAMP), adenosine 3′,5′-cyclic monophosphorothioate, Rp isomer (Rp-cAMP), and forskolin were purchased from EMD Chemicals (Gibbstown, NJ). Alexa 488 secondary antibody and TOPRO-3 were purchased from Invitrogen (Carlsbad, CA). All other chemicals were of the best quality available.

Isolation of primary rat BDE. Primary rat BDE were obtained from adult male Sprague-Dawley rats by immunosolation as described previously (24). This preparation results in a bile duct epithelial preparation that is ~98% pure as assessed by staining positively for the biliary epithelial markers gamma-glutamyl transpeptidase, cytokeratin-19, and cytokeratin-7 (23).

Isolation of IBDU and confocal microscopic detection of Ca2+ signals. Isolated bile duct units (IBDU) are spontaneously sealing isolated intrahepatic bile ducts that have been used widely in studies of bile ductular calcium signaling (2, 20). IBDU were isolated as described previously and plated onto glass coverslips (30, 33). Experiments were performed 24 h after plating, using only those bile duct units with visible, sealed lumens. IBDU were loaded with the Ca2+-sensitive dye fluo-4 AM. Coverslips containing the cells were transferred to a chamber on the stage of a Zeiss Axiovert microscope, perfused with HEPES-buffered solution containing ATP (100 μM) or forskolin (50 μM), and observed via a Zeiss LSM 510 confocal imaging system (Zeiss, Thornwood, NY). Serial images were obtained after perfusion. Fluo-4 fluorescence was excited by use of a krypton-argon laser at 488 nm; emitted fluorescence at >515 nm was collected. Changes in fluorescence over time were expressed as peak fluorescence divided by initial fluorescence.

Immunoblot detection of P2Y11. Immunoblot detection of P2Y11 was performed on primary rat BDE, and the following cell lines: H69 immortalized human cholangiocarcinoma cells, Mz-ChA-1 human cholangiocarcinoma cells, and 293T human embryonic kidney fibroblasts.

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Mz-ChA-1 cells were maintained in DMEM/F12 medium with 10% FBS, and HEK293T cells were maintained in DMEM medium with 10% FBS. H69 cells were maintained in standard growth media. Cells were lysed, and the lysates were separated by SDS-PAGE gel electrophoresis, and then transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA). Membranes were incubated with rabbit anti-P2Y11 antibody (Alomone Labs, Israel) diluted at 1:200 in 5% dry milk blocking buffer and then incubated with anti-rabbit secondary antibody. In some experiments, specificity of immunoblotting was determined by preincubation with P2Y11 control peptide provided by the manufacturer. Detection of bands was accomplished using ECL plus Western Blotting Detection System.

Confocal immunofluorescence for immunolocalization of P2Y11. Primary rat BDE were isolated as described above, plated on coverslips, and fixed in 4% parafomaldehyde in phosphate-buffered saline. Cell lines were plated and fixed as described for BDE. Slides were incubated with rabbit anti-P2Y11 (1:100) for 1 h at room temperature. Slides were then washed with PBS 3× then incubated with AlexaFluor 488-conjugated anti-rabbit antibody (Molecular Probes, Eugene, OR). Slides incubated with secondary antibody alone were used as a control for specificity of fluorescence detection. Confocal imaging of fixed cells was performed using a Zeiss LSM 510 confocal imaging system using krypton-argon and helium/neon lasers at ±630 magnification.

Real-time RT-PCR for detection of changes in IL-6 mRNA in rat BDE. Changes in IL-6 mRNA expression by BDE were determined by real-time RT-PCR using ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). Rat BDE were treated with ATP, ADP, AMP (negative control), UTP, UDP, suramin, or suramin + ATP (100 μM for each nucleotide; 50 μM suramin) for 2 h at 37°C. Total RNA was purified from cells by use of the RNAeasy Mini kit (Qiagen, Valencia, CA), and cDNA was synthesized by reverse transcriptase. The level of IL-6 was determined by using TaqMan probe, and VIC-labeled GAPDH probe (Applied Biosystems) was used as endogenous control for normalization. PCR was performed under the following parameters: 95°C × 10 min, then 40 cycles of 95°C × 15 s, 60°C × 60 s. In separate experiments, cells were treated with ATP, Rp-cAMP (100 μM), or ATP + Rp-cAMP. In further experiments cells were treated with ATP or ATP + db-cAMP (500 μM). In yet another set of experiments, cells were treated with thapsigargin (2 μM), db-cAMP forskolin (50 μM), or thapsigargin + forskolin. Changes in IL-6 mRNA were detected identical to the method described above.

Detection of IL-6 by ELISA. H69 cells were plated in a 48-well plate and incubated under the following conditions: buffer alone, BAPTA/AM (50 μM), Rp-cAMP (100 μM), ATPγS (100 μM), ATPγS + BAPTA/AM, or ATPγS + Rp-cAMP. After 2 h incubation, the cell supernatant was collected. IL-6 concentration was determined by using the Quantikine IL-6 immunoassay kit (R&D Systems, Minneapolis, MN) according to manufacturer instructions.

Detection of cAMP by ELISA. BDE were treated with buffer alone, AMP (negative control), ATPγS, or forskolin (positive control) for 2 h at 37°C. The reaction was stopped, and the cells were concentrated by centrifugation. Cells were then collected and resuspended in lysis reagent. Intracellular cAMP levels were detected using the cAMP Biotrak enzyme immunoassay (EIA) system (Amersham Biosciences, Piscataway, NJ) according to manufacturer instructions. In a separate set of experiments, BDE were treated with ATPγS (100 μM), BAPTA/AM (50 μM), or ATPγS + BAPTA. Changes in cAMP levels were determined as noted above.

Transfection of cells with IL-6 promoter constructs and detection of IL-6 promoter activity by luciferase assay. Rat IL-6 promoter truncations subcloned into pGL3Basic vector were kindly donated by Dr. Ernesto Canalis (University of Connecticut School of Medicine; Saint Francis Hospital, Hartford, CT). 293T cells were cultured in DMEM supplemented with 10% FBS. On the day prior to transfection, cells were split into 48-well plates and transfected with plasmids using FuGENE 6 (Roche Biosciences, Palo Alto, CA) according to manufacturer instructions. At the same time, control reporter vector pRL-SV40 was added to serve as a coreporter vector. At 24 h after transfection, cells were treated with thapsigargin (2 μM), db-cAMP (500 μM), forskolin (50 μM), thapsigargin + db-cAMP, or thapsigargin + forskolin overnight, and then cells were collected for assay. Cells were then collected and analyzed by a Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Luciferase activity was detected by use of a Synergy HT Multi-Detection Microplate Reader (BioTek, Winooski, VT). In separate experiments, control or mutant vectors (as described in the following section) were used for transfection, and cells were either untreated or treated with ATP or ATPγS (100 μM) overnight. Luciferase activity was assessed as determined above.

Site-directed mutagenesis of IL-6 promoter CRE. Analysis of the rat IL-6 promoter using the Transcription Element Search System (TESS, University of Pennsylvania Computational Biology and Informatics Biology Laboratory, www.cbil.upenn.edu/tess) confirmed the presence and location of the CRE of the rat IL-6 promoter (22, 38). Site-directed mutation of the CRE was performed by using the QuikChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). The following oligonucleotide primers were used: forward, 5′-TGG ATG CTA AAT AAG CTT ACA TTG TGC AAT-3′; reverse 5′-ATT GCA CAA TGT AAG CTT ACA TTG TGC AAT-3′. PCR amplification of mutants was performed using Pfu Turbo DNA polymerase (Stratagene) under the following parameters: 95°C × 1 min, then 18 cycles of 95°C × 15 s, 60°C × 5 s, 68°C × 6 min, then 68°C × 7 min. Successful mutation of mutant constructs was confirmed by sequencing prior to use.

RESULTS

ATP and ATPγS upregulate transcription and release of IL-6 by bile duct epithelia. The effects of extracellular nucleotides on BDE transcription of IL-6 were assessed by real-time RT-PCR. The pharmacological characterization of P2Y receptor subtypes may be found in Table 1. As seen in Fig. 1A, the P2Y11 ligand ATP, but not other nucleotides, upregulated IL-6 transcription. Interestingly, UTP and UDP, ligands of the rat P2Y2, P2Y4, and P2Y6 receptors (35), downregulated IL-6 transcription. The effect of ATP was inhibited by the P2Y inhibitor suramin. To further characterize the pharmacological characterization of this response, the relative effects of ATP and ATPγS were determined (Fig. 1B). The effect of ATPγS, a more potent synthetic agonist of P2Y11 (8), was greater than that of ATP. Taken together, these findings demonstrate that ATP and ATPγS upregulate IL-6 transcription. Pharmacological effects are compatible with activation of the P2Y11 receptor (6). The effect of ATPγS on release of IL-6 into the extracellular milieu was determined by ELISA in H69 immortal...
Expression of P2Y11 by BDE was determined by immunoblot against three human cell lines: H69 (immortalized human bile duct), Mz-ChA-1 (cholangiocarcinoma), and 293T (fibroblast). The greatest quantitative expression of the 34-kDa band was seen in 293T cells. In addition, rat lung and spleen tissue isolates also produced a single 34-kDa band (not shown). The 34-kDa band is the expected size protein product according to the manufacturer (Alomone Labs, http://www.alomone.com/p_postcards/database/334.htm). As seen in Fig. 3, C-E, confocal immunofluorescence of rat BDE shows rich plasma membrane and intracellular staining using the same P2Y11 antibody. Figure 3F shows that H69, Mz-ChA-1, and 293T cells express P2Y11 by immunofluorescence. H69 and Mz-ChA-1 P2Y11 fluorescence appears to be primarily found at the plasma membrane, whereas 293T P2Y11 fluorescence is found throughout the cell. This change in distribution in 293T cells is likely due to the primitive nature of these cells. Similar examples of proteins with restricted distribution expressed diffusely in cells early in embryonic development (26) or in dedifferentiated cancers (9) have been reported. Taken together, these data suggest that primary rat BDE and the cell lines used in this study express P2Y11 protein.

BDE upregulate cAMP levels in response to ATPγS. P2Y11 is unique among cloned P2Y receptors, since it induces increases in both intracellular Ca2+ and cAMP levels. Although the effects of extracellular ATP or other nucleotides on Ca2+ signals are well established (18, 29), the effects of nucleotides on cAMP signals on primary immunosolated rat BDE are unknown. As seen in Fig. 4A, ATPγS upregulated BDE cAMP levels to roughly the same level as forskolin (positive control), whereas AMP (negative control) had no effect. Thus ATPγS upregulates both Ca2+ and cAMP in BDE, which is compatible with the known signal transduction of P2Y11 (8). To demonstrate that ATPγS-sensitive upregulation of cAMP was not an artifact of Ca2+-sensitive adenyl cyclases (31, 42), a separate experiment was performed in the presence of the Ca2+ chelator BAPTA/AM. As seen in Fig. 4B, BAPTA had no effect on cAMP levels, suggesting that the ATPγS-dependent rise in cAMP was independent of Ca2+-sensitive adenyl cyclases.

Antibodies to human P2Y11 detect a distinct protein on immunoblot. Expression of P2Y11 by BDE was determined by immunoblot and confocal immunofluorescence (Fig. 2). As seen in Fig. 3A, immunoblot of rat BDE and the human cell lines Mz-ChA-1 and 293T with a commercial antibody to human P2Y11 produced a band of ~34 kDa in size. Furthermore, this band was undetectable after incubation of the antibody with P2Y11 peptide. Figure 3B shows that the 34-kDa band was obtained by using the same antibody in immunoblots against three human cell lines: H69 (immortalized human bile duct), Mz-ChA-1 (cholangiocarcinoma), and 293T (fibroblast). Changes in IL-6 protein release by H69 cells were determined by ELISA. ATPγS markedly upregulated IL-6 release (***p < 0.005 vs. control), and this was inhibited by BAPTA (**p < 0.001 vs. ATPγS). Interestingly, adenosine 3',5'-cyclic monophosphorothioate, Rp isomer (Rp-cAMP) had no effect on ATPγS-stimulated IL-6 release. BAPTA and Rp-cAMP alone were not statistically different from control (n = 3 for all conditions).
Exogenous increase of cAMP does not induce Ca^{2+} increases in IBDU. Recent reports demonstrate that under some physiological conditions cAMP-mediated secretion is dependent on purine-sensitive increases in cytosolic Ca^{2+} (20). To ensure that this effect did not distort the interpretation of the signal transduction studied in these studies, ATP- and forskolin-sensitive Ca^{2+} increases were studied in IBDU, which were used because they can be plated on glass coverslips for confocal video microscopic examination of Ca^{2+} signals (33). As seen in Fig. 5, ATP, but not forskolin, induced rises in cytosolic Ca^{2+}, demonstrating that cAMP-sensitive Ca^{2+} signals were not present under these conditions.

ATP upregulates IL-6 transcription by BDE in a manner dependent on cAMP and Ca^{2+}. The effects of the cAMP-dependent protein kinase inhibitor Rp-cAMP (3) on ATP-dependent increases in IL-6 transcription were assessed as shown in Fig. 6A. Rp-cAMP blocked the increase in IL-6 transcription due to ATP. Rp-cAMP alone decreased IL-6 transcription below control, suggesting that baseline transcription of IL-6 by BDE is cAMP dependent. Furthermore, addition of the synthetic cAMP analog db-cAMP further increased the effect of ATP on IL-6 transcription (Fig. 6B). The effects of the cell-permeant intracellular Ca^{2+} chelator BAPTA/AM (21, 34) on ATPγS-dependent increases in IL-6 transcription were assessed as shown in Fig. 7. BAPTA blocked the increase in IL-6 transcription due to ATPγS. Interestingly, BAPTA alone increased IL-6 transcription above control, suggesting that Ca^{2+} has a role in the regulation of baseline IL-6 transcription. Taken together, these data demonstrate that the upregulation of IL-6 transcription by ATP is mediated by cAMP and Ca^{2+}. To
determine whether cAMP and/or cytosolic Ca\(^{2+}\) independently or synergistically regulated bile ductular IL-6 mRNA levels, BDE were treated with forskolin, thapsigargin, or forskolin + thapsigargin. As seen in Fig. 8, thapsigargin but not forskolin upregulated IL-6 mRNA, and forskolin did not upregulate IL-6 mRNA beyond the level induced by thapsigargin. Since thapsigargin potently upregulates cytosolic Ca\(^{2+}\) in a manner differently than G protein-coupled hormones, these data suggest that massive increases in cytosolic Ca\(^{2+}\) may supersede hormonal increases in cAMP and Ca\(^{2+}\) typical of those induced by ATP\(\gamma\)S.

**The IL-6 promoter is regulated by cAMP and Ca\(^{2+}\) via the cAMP-response element.** A truncation of the cloned rat IL-6 promoter known to express the CRE was expressed in 293T cells, and promoter activity was assessed by dual-reporter luciferase assay. As seen in Fig. 9, thapsigargin, which releases stored intracellular Ca\(^{2+}\) via nonhormonal means (32), upregulated IL-6 promoter activity. Neither the cAMP analog db-cAMP nor the adenyl cyclase activator forskolin (30) significantly upregulated IL-6 promoter activity above baseline. However, both db-cAMP and forskolin upregulated IL-6 transcription of the IL-6 promoter above the increase induced by thapsigargin. This finding is distinct from that in IL-6 mRNA levels in BDE (Fig. 8) and may be due to intrinsic differences between cell types. The role of CRE in this upregulation was determined by site-directed mutagenesis of the CRE in the IL-6 promoter (Fig. 10). Mutation of the CRE blunted IL-6 promoter sensitivity to both forskolin (Fig. 10A) and thapsigargin (Fig. 10B). Furthermore, IL-6 promoter truncation not contain-
ing the CRE had no IL-6 promoter activity above empty vector alone. Taken together, these data demonstrate that the IL-6 promoter is activated by both cAMP and Ca\textsuperscript{2+} and that this activation is mediated by CRE.

**Extracellular ATP upregulates IL-6 promoter activity via CRE.** The effects of ATP and on IL-6 promoter activity are shown in Fig. 11. Of note, 293T cells express the P2Y\textsubscript{11} receptor (Fig. 3). Similar to the effects noted in Fig. 7, mutation of the CRE abrogated ATP-sensitive increases in IL-6 promoter activity. Furthermore, IL-6 promoter truncation not containing the CRE had no IL-6 promoter activity above empty vector alone. This experiment demonstrates that the IL-6 promoter is activated by extracellular ATP and ATP\textsubscript{S} and that this activation is mediated by CRE.

**DISCUSSION**

Although IL-6 is classified as an inflammatory cytokine, its effects on the liver are quite varied. In fact, increasing evidence demonstrates that IL-6 is not only beneficial but is in fact necessary for survival in response to hepatic injury. Loss of either IL-6 or its receptor gp130 leads to decreased survival and hepatic health in a variety of forms of liver injury (11, 19, 27). IL-6 is of particular importance for survival of BDE and liver progenitor cells, which either are or are closely related to BDE (1, 37). IL-6 may be connected to BDE survival in two ways. First, IL-6 directly upregulates BDE proliferation (40). Second, as our group has shown, IL-6 potently downregulates transcription and expression of the ectonucleotidase NTPDase2 by PF. Loss of NTPDase2, in turn, uncouples purinergic signals at the basolateral aspect of BDE, leading to upregulation of BDE proliferation. This mechanism is of critical importance in the upregulation of BDE in biliary cirrhosis both in rats and humans (16, 24). Thus, in the liver, IL-6 may be regarded as a proliferative signal, rather than or in addition to its role as a mediator of inflammation.

**Fig. 6.** Upregulation of IL-6 transcription by ATP is cAMP dependent. A: effect of Rp-cAMP on IL-6 transcription. IL-6 transcription was determined by real-time RT-PCR as described above. As seen in Fig. 1, ATP upregulated IL-6 transcription (**P < 0.02 vs. control). Rp-cAMP reduced IL-6 transcription (**P < 0.02 vs. control) and inhibited the increase in IL-6 transcription by ATP (**P < 0.02 vs. ATP) (n = 3 for each condition). B: effect of dibutyryl-cyclic AMP (db-cAMP) on IL-6 transcription. IL-6 transcription was determined as above. ATP upregulated IL-6 transcription (**P < 0.05 vs. control), and the addition of db-cAMP upregulated IL-6 transcription beyond this level (**P < 0.001 vs. control) (n = 8 for each condition).

**Fig. 7.** Upregulation of IL-6 transcription by ATP is Ca\textsuperscript{2+} dependent. IL-6 transcription was determined by real-time RT-PCR as described in Fig. 1. BAPTA/AM increased IL-6 transcription (**P < 0.002 vs. control). As seen in Fig. 1, ATP\textsubscript{S} increased IL-6 transcription (**P < 10\textsuperscript{-5} vs. control), and BAPTA/AM inhibited the increase in IL-6 transcription by ATP\textsubscript{S} (**P < 10\textsuperscript{-5} vs. ATP\textsubscript{S}) (n = 6 for each condition).

**Fig. 8.** Nonhormonal Ca\textsuperscript{2+} but not cAMP increases regulate IL-6 upregulation. IL-6 transcription was determined by real-time RT-PCR as described in Fig. 1. Thapsigargin increased IL-6 transcription (**P < 0.02). Forskolin did not alter IL-6 transcription (**P = NS), and the addition of forskolin to thapsigargin had no effect on the increase induced by thapsigargin (**P = NS vs. thapsigargin; n = 3 for all conditions).


The concept of release of inflammatory cytokines such as IL-6 by cells outside of the immunological or reticuloendothelial systems is relatively new. However, release of IL-6, IL-8, and monocyte chemoattractant protein-1 (MCP-1/CCL2) by epithelia without immune specialization has been increasingly documented. Most applicable to this manuscript, BDE are known to release IL-6, and they have been shown to upregulate IL-6 release in conditions ultimately leading to biliary cirrhosis. Here we provide a discrete mechanism by which this occurs (although other mechanisms may exist as well). Cells as distinct as nasal epithelia (5), airway epithelia (14), and corneal epithelia (4) release inflammatory mediators and upregulate these in response to stress. Additionally, we have previously demonstrated that BDE upregulate release of MCP-1 after common bile duct ligation, leading to myofibroblastic differentiation of PF (25), suggesting that BDE in particular may employ regulated release of inflammatory mediators as a survival and wound-healing signal. Together, these data suggest that epithelia respond to injury via release of inflammatory cytokines, which may in turn direct such processes as immune cell recruitment, fibrosis, and cell proliferation.

A specific question addressed in this manuscript is the mechanism of transcriptional regulation of the IL-6 promoter. Here we show a mechanism suggesting that activation of a purinergic receptor functionally similar to the cloned human P2Y1 receptor leads to increases in cAMP and Ca2+, both of which act at the IL-6 promoter CRE site to upregulate IL-6 transcription. Purinergic upregulation of the IL-6 promoter has also been demonstrated in airway epithelia, although the mechanism shown was distinct, involving the P2Y2 receptor, Ca2+, and p38 MAPK (14). Although a promoter element regulating this effect was not defined, upregulation of proliferation was noted as early as 2 h, which was the shortest time point noted in our studies as well. Studies examining promoter elements regulating the transcription of IL-6 have identified several important promoter elements, including those regulated by CRE, activator protein-1, nuclear factor for IL-6, and NF-κB (22). Interestingly, in renal mesangial cells, the greatest upregulation of IL-6 requires the presence of cAMP, suggesting a particular role for CRE in this process (22). A similar cAMP-dependent mechanism has also been shown in intestinal epithelium-like T84 cells (38), supporting the importance of this mechanism. Thus, although the IL-6 promoter expresses several cis-acting promoter elements, the CRE may be of special importance in epithelia.

We propose that the relevance of this work is its identification of a novel pathway that may be of particular importance in hepatic survival in response to injury. Since an insufficient proliferative response impairs survival after injury (especially biliary injury), the elucidation of pathways leading to bile...
ductular proliferation is critical. Ultimately this work may lead to identification of novel pharmacological approaches in such diverse conditions as biliary cirrhosis, patients who have undergone partial hepatectomy, and those who have undergone liver transplantation.

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