Specific Inhibition of c-Raf Activity by Semapimod Induces Clinical Remission in Severe Crohn's Disease

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There is a substantial need for novel treatment strategies in Crohn’s disease (CD), a chronic relapsing inflammatory disease of the gut. In an earlier study, we reported clinical efficacy of a 2-wk treatment with semapimod (CNI-1493) in 12 patients with therapy resistant CD. The aim of this study was to identify the cellular target underlying semapimod action. In vitro experiments with murine macrophages showed impaired MAPK signaling and decreased cytokine production due to semapimod treatment. In vitro kinase assays revealed c-Raf as a direct molecular target of semapimod, and semapimod did not affect b-Raf enzymatic activity. Immunohistochemistry performed on paired colon biopsies obtained from CD patients (n = 6) demonstrated increased expression of phospho-MEK, the substrate of Raf. Strikingly, phospho-MEK levels were significantly decreased in patients with a good clinical response to semapimod, but no decrease in phospho-MEK expression was observed in a clinically nonresponsive patient. In conclusion, this study identifies c-Raf as a molecular target of semapimod action and suggests that decreased c-Raf activity correlates with clinical benefit in CD. Our observations indicate that c-Raf inhibitors are prime candidates for the treatment of CD.


Inhibitors of intracellular signaling pathways have proven effective in a wide range of experimental inflammatory disorders, including experimental colitis (1). Small molecules targeting these signaling cascades are generally considered as a promising novel strategy for the clinical management of inflammatory bowel diseases (i.e., Crohn’s disease (CD)) and ulcerative colitis. In particular, pharmaceutical intervention of the MAPK pathways of intracellular signaling mediators attracts widespread interest (2–6). Three major MAPK cascades have been identified: ERK, JNK, and p38 MAPK, and these pathways are critically involved in inflammatory pathology, including CD (6–8). Selective MAPK inhibitors targeting the p38 MAPK, ERK, and JNK pathway demonstrated anti-inflammatory effects in preclinical models (1, 9–13). Despite the fact that the impact of MAPK pathways on inflammatory pathology is profound, the molecular details of these signaling cascades in the pathogenesis of inflammatory disorders and their possible therapeutic value remain to be elucidated. In view of the redundancy of MAPK pathways and the extensive cross-talk between these and other routes of signal transduction (e.g., NF-κB), such information is of great importance. We have reported that treatment of therapy resistant CD patients with the small molecule semapimod resulted in a reduction of disease activity and induction of clinical remissions (14). Although it has been demonstrated that semapimod interferes with the phosphorylation of both p38 and JNK (14), the exact underlying molecular mechanism of semapimod action remains to be characterized. The identification of the molecular target of semapimod has important clinical relevance because it may prompt synthesis of a novel class of anti-inflammatory compounds. In this study we have identified macrophages as the target cells of semapimod action, and we characterized c-Raf as the molecular target. Reduced expression of phospho-MEK, a downstream target of c-Raf, in colon biopsies correlated with clinical benefit in semapimod-treated CD patients. In contrast, no reduced phospho-MEK was observed in mucosal biopsies obtained from a nonresponder. These results indicate that c-Raf activity is a critical mediator of disease progression in CD, and identify c-Raf as a novel therapeutic target for the clinical management of CD.

Materials and Methods

Abs and reagents

Phospho-specific Abs directed against p38 Thr180/Tyr182, ERK1/2 Thr202/Tyr204, MEK1/2 Tyr202/204, c-Raf Ser338, stress-activated protein kinase/JNK Thr183/Tyr185, p21-activated protein kinase (PAK)1/2 Thr382/385, SEK1/MAPK kinase (MKK)4 Thr202, MKK3/6 Ser180/183, and anti-mouse, and rabbit anti-goat were from Santa Cruz Biotechnology. HRP-conjugated goat anti-rabbit, goat anti-mouse, and rabbit anti-goat were from DakoCytomation, and semapimod (CNI-1493) was acquired from Cytokine PharmaSciences (batch date 3/13/2004; lot no. 08610302). The anti-CD68 mAb was from DakoCytomation, and anti-CD14 mAb was obtained from BD Biosciences. Anti-human CD3 (CD3ε) mouse was kindly provided by Dr. A. te Velde (Academic Medical Center, Amsterdam, The Netherlands). Anti-CD28 was from Sanquin. The c-Raf and b-Raf kinase kits were obtained from Upstate Biotechnology.

CD4 purification and cell sorting

PBMC were isolated from whole blood of healthy volunteers by Ficoll-Isoaque density gradient centrifugation (Amersham Biosciences). The monocytes present in the PBMC pellet were removed by an adherence procedure: cells were plated out in 6-well plates (Cellstar; Greiner Bio-One) at a final concentration of 5 × 10^6 cells/well for 1.5 h at 37°C, and...
subsequently, nonadherent cells were harvested for magnetic cell sorting. CD4+ T cells were purified by depletion of non-CD4+ T cells (negative selection) using the MACS system. Non-CD4+ cells were indirectly magnetically labeled with a mix of biotin-conjugated mAbs (against CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCR γδ, and glycoporphin A) bound to MicroBeads conjugated to a monoclonal anti-biotin Ab, as secondary labeling agent (Miltenyi Biotec). The magnetically labeled non-CD4+ T cells were depleted by retaining them on a MACS Column in the magnetic field of the autoMACS Separator (Miltenyi Biotec), whereas the unlabeled fraction of CD4+ T cells passed through the column. The sample purity was assessed by FACS (BD Biosciences) with PE-conjugated CD4 and FITC-conjugated CD3 mAbs (BD Biosciences) (purity >95% CD3+CD4+; data not shown).

Generation of dendritic cells (DC)

DC generation from PBMC (obtained from healthy volunteers) was performed as previously described (15, 16). Briefly, PBMC were resuspended in Adoptive Immunotherapy Media (Invitrogen Life Technologies), and allowed to adhere to 6-well plates (Cellstar; Greiner Bio-One). After 2 h at 37°C, nonadherent cells were removed and the adherent cells were cultured in medium supplemented with 50 ng/ml GM-CSF and 1000 U/ml IL-4. Next, monocytes were incubated for 6 days in X-VIVO 15 medium (Bio-Whittaker) supplemented with 1000 U/ml GM-CSF (Berlex) and 1000 U/ml IL-4 (BD Biosciences). The immature DC were stimulated at day 6 in X-VIVO 15 medium supplemented with a cytokine mix containing TNF-α (10 ng/ml), PGE2 (1 µg/ml), IL-1β (10 ng/ml), IL-6 (150 ng/ml), GM-CSF (800 U/ml), and IL-4 (500 U/ml). After 24 h, mature DC were harvested for phenotyping using a panel of mAbs and analyzed on a FACSscan with CellQuest software (BD Biosciences), as previously described (17).

Cell culture

4/4 macrophages (murine), which are phenotypically and functionally not different from primary isolated mature macrophages (18, 19), were cultured in RPMI 1640 (Invitrogen Life Technologies), supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, and penicillin-streptomycin (“complete”) in a humified 5% CO2 environment at 37°C. Human CD4+ T cells were grown in IMDM (Invitrogen Life Technologies), supplemented with 10% FCS, 2 mM L-glutamine, and penicillin-streptomycin (“complete”) in a humified 5% CO2 environment at 37°C.

MTT viability assay

The cytotoxic effect of semapimod was studied in macrophages, which were incubated overnight with increasing concentrations of semapimod (0.01, 0.1, 1, 10, and 100 µM diluted in medium) with or without LPS (100 ng/ml). Cell viability was assessed by MTT colorimetric assay. After overnight incubation, 0.5 mg/ml MTT was added to the medium for 1–2 h at 37°C, and subsequently isopropanol/0.04 N HCl was added. The OD560 was determined using an ELISA plate reader (Bio-Rad). Treatment with semapimod concentrations ≤ 1 µM did not affect cell viability. A semapimod-induced cytotoxic effect was observed at semapimod concentrations > 1 µM (10 and 100 µM; data not shown).

Cytokine bead array (CBA)

Macrophages and CD4+ T cells were pretreated for 1 h with various concentrations of semapimod and cultured up to 24 h with either LPS (100 ng/ml) or anti-CD3 (immobilized on plastic) and anti-CD28 (3 µg/ml, soluble) Abs, respectively. Furthermore, mature DC were pretreated for 1 h with 0.1 and 1 µM semapimod. Medium was removed, and cells were cultured for 24 h in fresh medium containing CD40L transduced J558 cells (1:1). The CD40L transduced mouse plasmacytoma cell line (J558), was a kind gift from Dr. P. Lane (University of Birmingham, Birmingham, U.K.) (20). Cytokine levels were analyzed in supernatants of macrophages, DC, and T cells by CBA (BD Biosciences) using a flow cytometer (BD Biosciences), according to routine procedure.

Western blot analysis

MAPK signaling pathways were studied on Western blot using phospho-specific Abs against a variety of MAPK signal transduction molecules. Macrophages were seeded in 6-well plates at a final concentration of 1 × 106 cells/well and grown overnight. Cells were pretreated for 1 h with 0.1 and 1 µM semapimod and subsequently stimulated with LPS (100 ng/ml) for 15 min. After washing with PBS, cells were harvested in sample buffer (150 mM Tris-HCl, 6% SDS, 3% 2-ME, 20% glycerol, and 1 mg of bromophenol blue (pH 6.8)), and whole cell lysates were loaded on 10% SDS-PAGE and subsequently transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore). Membranes were blocked with 1% Protifir in TBST (0.05 M Tris, 150 mM NaCl, and 0.05% Tween 20). Primary and secondary HRP-conjugated Abs were diluted in 1% Protifir in TBST, and proteins were visualized using the Lumi-Light substrate (Roche). Blots were incubated in stripping buffer (62.5 mM Tris-HCl (pH 6.8), 100 mM 2-ME, and 2% SDS) for 1 h at 50°C and subsequently reprobed with appropriate Abs to evaluate for equal loading. In addition, T cells (overnight cultured in 6-well plates, 3 × 106 cells/well; pretreated with semapimod (1 h, 0.1 and 1 µM) and subsequently activated with anti-CD3/anti-CD28 Abs (15 min) were analyzed on Western blot using phosphospecific Abs.

Raf in vitro kinase assays

Raf in vitro kinase assays were used according to the instructions of the manufacturer (Upstate Biotechnology). Truncated constitutively active Raf (b-Raf and c-Raf) was diluted in a Mg/ATP mixture and reaction buffer, and incubated on ice with semapimod (1 µM) for 5 and 10 min. Next, recombinant inactive MEK was added and in vitro kinase assays were performed at 30°C for 20 min. Active Raf together with MEK and Raf without MEK served as a positive and negative control, respectively. Samples were dissolved in sample buffer, incubated at 95°C for 5 min, and analyzed on Western blot using an anti-phosphoMEK Ser218/222/MEK2 Ser222/223 Ab.

Immunohistochemistry

To assess the amount of active MEK in the intestinal mucosa, screening and week 4 colon specimens were obtained from most affected regions of inflammation of CD patients (n = 6) who participated in the semapimod (CNI-1493) study (14). Biopsies were analyzed for phospho-MEK expression. Paraffin sections (4 µm) were dewaxed and rehydrated in graded alcohols, and endogenous peroxidase activity was quenched with 1.5% H2O2 in methanol (15 min, room temperature). Ag retrieval was performed by heating for 10 min at 100°C in 0.01 M sodium citrate. After washing (PBS), nonspecific staining was reduced by a blocking step with 10 mM BSA. Slides were incubated for 1 h at 4°C with an anti-phospho-MEK (Ser218/222/MEK2 Ser222/223) Ab diluted in 1% BSA 0.1% Triton X-100. Slides were incubated with an anti-rabbit IgG (H+L) peroxidase-linked secondary Ab (800 U/ml). Peroxidase activity was detected using diaminobenzidine (Fast DAB; Sigma-Aldrich) in 0.05 M Tris (pH 7.4). Sections were briefly counterstained with hematoxylin (Mayer’s; Fluka) when appropriate, dehydrated in graded alcohols, and mounted with Pertex (His-tolub Products) under coverslips. Controls consisted of omitting the primary and secondary Ab and use of an appropriate Ig control (data not shown).

Statistical analysis

Quantitative confirmation came from experiments in which the number of phospho-MEK-positive cells was counted in sections in a blinded fashion. Two pictures of each section were taken at ×200 magnification, and positive cells were counted, blind to treatment and day of endoscopy in each microscope field with the use of an image analysis program (EFM Software). Pictures appeared randomly on a computer monitor and all intensely staining cells were marked positive by an observer, counted, and stored by the image analysis program for later data analysis. Statistical analysis was performed by use of the Wilcoxon test, and a value of p < 0.05 was considered as statistically significant.

Results

Semapimod does not affect MAPK signaling cascades in T cells

It has been previously reported that T cell cytokine production is not influenced by semapimod (21), and this was confirmed in our laboratory (data not shown). ERK, JNK, and p38 MAPK signal transduction pathways were activated in T cells stimulated with anti-CD3, anti-CD28 Abs, and this was not affected by incubation with semapimod (Fig. 1). Thus, these findings indicate that T cells are not direct target of semapimod action.
Semapimod does not influence IL-12 cytokine production in activated mature DC

Recently, it has been reported that semapimod interferes with DC maturation (22), which prompted us to study the effect of semapimod on mature DC. The effect of semapimod on IL-12 cytokine production was studied in CD40L-activated mature DC because it is generally accepted that this is an important Th1 differentiation mechanism that is relevant for CD (23–26). Our data indicate that semapimod does not interfere with IL-12 cytokine production in activated mature DC, suggesting that this compound does not influence the capacity of DC to induce a Th1-type response (Fig. 2).

Semapimod inhibits cytokine production in macrophages

To determine the usefulness of in vitro stimulation of macrophages for studying the underlying molecular mechanism of semapimod action, the effect of semapimod on cytokine production in macrophages was investigated. Semapimod treatment resulted in a dose responsive reduction of LPS-induced TNF-α, IL-1β, and IL-6 protein levels (Fig. 3). Decreased cytokine production observed upon treatment with 0.01, 0.1, and 1 μM semapimod was not a result of reduced cell viability, as MTT colorimetric assays revealed significant cytotoxicity of semapimod only at concentrations of 10 and 100 μM (data not shown). These observations confirm that semapimod effectively blocks cytokine synthesis in macrophages (27–29) and indicate that incubation of macrophages with semapimod concentrations of 0.1 and 1 μM constitute an appropriate
Semapimod inhibits MAPK signaling pathways in macrophages
As it has been previously shown that semapimod blocks p38 MAPK and JNK phosphorylation in vitro (14), this prompted us to study the effects of semapimod on MAPK signaling pathways in more detail. Therefore, the activation status of various kinases involved in MAPK signaling was analyzed by immunoblotting semapimod-treated macrophages using phosphospecific Abs against MAPK signal transduction molecules. LPS enhances phosphorylation of JNK, ERK, and p38 MAPKs and semapimod treatment resulted in suppressed phosphorylation of all MAPKs (i.e., JNK, ERK, and p38) (Fig. 4). LPS-induced phosphorylation of upstream MAPK activators was observed (i.e., MEK1/2 for ERK, MKK4 for JNK, and MKK3/6 for p38 MAPK). Impaired phosphorylation of MAPK kinases (i.e., MEK1/2, MKK4, and MKK3/6) was seen upon pretreatment with semapimod. c-Raf phosphorylation was observed in stimulated and control cells (no LPS), in concordance with a previous report (32). Semapimod did not affect c-Raf phosphorylation, nor did it affect phosphorylation of PAK, an upstream c-Raf activator (33). These in vitro data suggest that semapimod interferes with MAPK activation upstream from MAPK kinase and downstream from PAK, thereby making c-Raf a likely candidate target.

c-Raf is a molecular target of semapimod
The observed inhibition of MEK phosphorylation by semapimod in LPS-stimulated macrophages without an apparent accompanying effect on c-Raf activation itself may indicate that semapimod is a direct inhibitor of c-Raf catalytic activity. To directly test this hypothesis, we used two protein in vitro kinase assays in which the activity of recombinant constitutively active Raf to phosphorylate MEK was tested in the presence or absence of semapimod. Incubation of active c-Raf or b-Raf together with MEK in the absence of semapimod clearly induced MEK phosphorylation. Pretreatment of c-Raf with 1 μM semapimod for 5 and 10 min abolished its potential to phosphorylate MEK in this two-protein assay (Fig. 5). Importantly, semapimod treatment of b-Raf, an enzyme that is closely related to c-Raf, did not result in altered MEK phosphorylation (Fig. 5), demonstrating the specificity of semapimod as an inhibitor of c-Raf enzymatic activity. These data reveal specific and direct inhibition of c-Raf enzymatic activity by semapimod.

Semapimod inhibits c-Raf activity in vivo
In an earlier study, patients with severe CD (mean CD Activity Index (CDAI) of 380 points) received either 8 or 25 mg/m² semapimod i.v. once daily for 12 consecutive days (14). Paired colon biopsies were available at baseline and after 4 wk of treatment for six CD patients. Their mean age was 32 years, two were male, five were treated with infliximab (anti-TNF), two were treated with steroids, and one was treated with mesalazine before semapimod treatment. Three patients received 8 mg/m² semapimod, and the remaining three were treated with a 25 mg/m² dose. Clinical response was defined by a CDAI reduction of ≥25% and ≥70 points compared with baseline or the occurrence of a clinical remission as assessed by a reduction of CDAI of <150 points (34). Clinical response was observed in five of six patients (mean CDAI reduction of 261 points at week 16), of whom four went into clinical remission at 16 wk after initiation (Fig. 6). One of six patients did not respond to semapimod treatment. The observed clinical response rate correlated to a decrease in C-reactive protein (CRP) serum concentrations: all responders (five patients) demonstrated decreased CRP levels and the single patient that did not show a decrease of the serum CRP did not respond clinically (Fig. 6).

To establish the effect of semapimod treatment on c-Raf activity in vivo, colon biopsies were analyzed for phospho-MEK expression. Neutrophils and monocytes were detected as CD14+ cells (Fig. 7E) and macrophages as CD68+ cells (Fig. 7F) in adjoining sections. Lymphocytes (identified as CD3+ cells) had a different distribution pattern compared with phospho-MEK-positive cells (data not shown). Immunohistochemical analysis revealed high levels of phospho-MEK at baseline, which was mainly localized to
macrophages and neuroendocrine cells in the crypts (Fig. 7, A and C). Faint phospho-MEK staining was seen in the neutrophil and monocyte compartment. The decrease in phospho-MEK expression after therapy, observed in five of six patients, was statistically significant (p < 0.0348) (Fig. 7G). One patient did not show decreased phospho-MEK expression (Fig. 7D). Interestingly, the nonresponder did not demonstrate decreased phospho-MEK expression (Fig. 7D). Subsequently, we analyzed whether the reduction of phospho-MEK-positive cells correlated with clinical outcome (defined by CDAI and CRP levels). Interestingly, the nonresponder did not demonstrate decreased phospho-MEK expression in colon biopsies obtained at week 4 after treatment (Fig. 7D) compared with baseline (Fig. 7C). In contrast, all responders revealed significant reduced numbers of phospho-MEK-positive cells after therapy (Fig. 7H). These data indicate that semapimod inhibits c-Raf activity not only in vitro but also in vivo.

Discussion
As a consequence of the limited efficacy and significant toxicity of current therapy, there is widespread interest in the development of novel drugs for the clinical management of CD. We have reported that semapimod, in a small and uncontrolled clinical trial in severe CD patients, seemed to have significant clinical benefit and that clinical responses correlated with an inhibitory effect on the p38 MAPK and JNK signaling cascades (14). Despite this therapeutically relevant outcome, the molecular mechanism of semapimod action remains unexplained. We here report that c-Raf in macrophages is the molecular target of semapimod: our studies with LPS-stimulated macrophages show that this molecule inhibits LPS signaling at the level of c-Raf, resulting in reduced proinflammatory cytokine production. Furthermore, semapimod pretreatment blocked MEK phosphorylation (a Raf substrate) by inhibiting c-Raf in a two-protein in vitro kinase assay, whereas the enzymatic activity of b-Raf (which is structurally closely related to c-Raf) was not influenced by semapimod. Thus, semapimod is a highly specific c-Raf inhibitor.

In agreement with a role for semapimod as an in vivo inhibitor of c-Raf, colon biopsies obtained from semapimod-treated CD patients who responded to therapy showed significant decreased phospho-MEK expression, which was predominantly localized to macrophages and neuroendocrine cells. Interestingly, whereas semapimod is highly active in the macrophage compartment, our in vitro data confirm an earlier report that T cells are not direct...
target cells of semapimod action (21). Semapimod treatment did not affect cytokine production nor did it affect MAPK signaling cascades in T lymphocytes. A likely explanation may be found in the relative importance of b-Raf in comparison to c-Raf in activating MAPK cascades in lymphocytes, further emphasizing the specificity of the c-Raf inhibitory effect observed (35–37). We also evaluated whether semapimod could affect IL-12 cytokine production in activated mature DC, a major pathogenic mechanism in CD4⁺ lymphocyte-mediated pathology, such as CD (23–26). Semapimod did not interfere with IL-12 cytokine production, suggesting that this compound does not influence Th1-mediated responses by mature DC in vitro. Taken together, these observations indicate that the cell-specific effects of semapimod are related to Raf isotype specificity and hypothesize that c-Raf inhibition in macrophages is the primary effector of semapimod action in CD.

Macrophages play a major role in initiating, amplifying, and perpetuating the inflammatory response by activating immune cells, including monocytes and T cells (38, 39). However, we are not aware of data indicating that a therapeutic strategy that mainly targets macrophages has therapeutic efficacy in a chronic inflammatory disease in humans. Our current data strongly suggest that semapimod-induced inhibition of c-Raf in one particular immune cell (macrophage) results in a clinical response in severe CD, independent from an effect on T cells or DCs. Hence, the present
study provides novel evidence for a pivotal role of macrophages in the pathogenesis of CD (40–45). The identification of c-Raf as the molecular target of semapimod raises questions regarding the function of this molecule in the inflammatory process. Previous work has demonstrated that c-Raf is involved in inflammatory mechanisms by controlling downstream signaling molecules such as the proinflammatory transcription factor NF-κB (46–48), thereby mediating cytokine synthesis and other proinflammatory mediators (49–55). In addition, various studies have identified c-Raf as an important antiapoptotic molecule and its inhibition may well cause effector macrophages to undergo programmed cell death in the proinflamatory inflammatory environment present in the gut of CD patients (56, 57). As a result, induced apoptosis of macrophages could lead to an attenuation of the inflammatory process. Further studies investigating apoptosis in the gut of semapimod-treated CD patients may provide answers to this important question. Our data indicate that the proinflammatory effects of c-Raf include not only activation of ERK, but also JNK and p38, and thereby mediating cytokine synthesis and other proinflammatory small molecules.

Clinical studies with semapimod demonstrated that the drug is relatively well tolerated (14, 30, 58). Side effects included local irritation at the infusion site (phlebitis) and mild increases in liver enzymes, both resolving spontaneously within weeks. Preliminary analysis of a large controlled study with semapimod in moderate to severe CD did not detect clinical benefit. This result is probably largely due to the short exposure period (3–5 days), which was allowed in this study design (59).

Various Raf inhibitors have passed phase III as anticancer strategy showing a tolerable safety profile (60–68). To our knowledge, largely due to the short exposure period (3–5 days), which was down-regulated by severe CD did not detect clinical benefit. This result is probably largely due to the short exposure period (3–5 days), which was allowed in this study design (59).

References


